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14. ABSTRACT
How prostate tumors become metastatic is virtually unknown. A prostate metastasis suppressor gene, KAI1/CD82, known to associate with laminin receptors, allowed us to test the hypothesis that loss of KAI1/CD82 expression alters the function of laminin integrins in prostate cancer cells, resulting in altered intracellular signaling and increased invasion leading to metastasis. We have demonstrated that in metastatic tumor cells, where elevated c-Met expression and activation by integrins is responsible for enhancing laminin-dependent migration and invasion, re-expression of CD82 suppresses, while loss of CD82 enhances c-Met activation. Orthotopic injection of CD82-expressing metastatic cells into mouse prostates suppresses both metastasis and growth in vivo. CD82 appears to regulate c-Met activation by altering the distribution of c-Met on the cell surface, possibly through CD82 association with another tetraspanin, CD9, and their joint association with β1 integrin. Our studies have advanced the knowledge of how members of the tetraspanin family function and are potentially applicable to all metastatic disease, since KAI1/CD82 loss has been reported in many types of cancers. Our results suggest that targeting c-Met would be a logical approach to therapeutic intervention of metastatic disease. Our findings further suggest that together CD82 expression and c-Met activation could be used as a potential biomarker pair for the prediction of metastatic disease. In addition we have shown that the PI-3K/Akt pathway is critical for laminin-specific survival in metastatic prostate cells, but activation of the androgen receptor (AR) by-passes the need for PI-3K signaling when cells are adherent to laminin. Since over 90% of metastatic prostate cancers still express AR, targeting the PI-3K pathway alone would not be sufficient to kill tumors in a laminin-rich environment.

15. SUBJECT TERMS
Cell biology, integrins, KAI1/CD82, tetraspanins, metastasis, androgen, signal transduction, 3-D culture

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INTRODUCTION

Prostate cancer death is primarily due to metastasis. Surprisingly, we understand very little about how prostate tumors become metastatic. The most frequently observed mechanism for tumor cell exit from the prostate is through interactions with nerve fibers and perineural migration out of the organ. Nerve fibers are surrounded by the extracellular matrix protein, laminin, and thus it has been proposed that for prostate tumor cells to exit the gland and metastasize they must acquire the ability to migrate on laminin. Adhesion and migration on laminin is mediated by two specific laminin receptors, $\alpha6\beta1$ and $\alpha3\beta1$. Interestingly, elevated expression of these two integrins is highly correlative with the invasive and metastatic phenotype of prostate cancer. The recent identification of a prostate tumor suppressor gene, KAI1/CD82, suggests a molecular mechanism by which migration on laminin and exit from the prostate during metastasis might be achieved. KAI1/CD82 is known to associate with the $\alpha6\beta1$ and $\alpha3\beta1$ laminin receptors. Therefore, **our hypothesis** is that loss of KAI1/CD82 expression alters the function of $\alpha3\beta1$ and $\alpha6\beta1$ integrins in prostate cells resulting in altered intracellular signaling, decreased tissue organization, and increased cell motility leading to increased metastatic potential. Metastatic disease is also associated with progression to androgen independence. One possibility is that androgen independence occurs through changes in growth factor signaling that favor increased growth and survival. Studies in our laboratory indicate that growth factor signaling is regulated by integrins. Thus it is possible that the changes in integrin function, due to loss of KAI1/CD82, alters androgen responsiveness. The loss of expression of a gene, such as KAI1/CD82, is hard to target therapeutically because it requires a mechanism for replacing the gene. However, if we can identify a downstream effector that is up-regulated, then our ability to address this genetic defect is increased by our ability to design an inhibitor to that downstream response. Therefore, it is important not only to identify genetic changes, but to also characterize the biological effects of the genetic changes.

BODY

Our working hypothesis is that loss of KAI1/CD82 alters the expression and/or function of $\alpha3\beta1$ and $\alpha6\beta1$ integrins in prostate cells resulting in altered intracellular signaling, decreased tissue organization, and increased cell motility leading to increased metastatic potential. Alterations in integrin signaling and function, due to loss of KAI1/CD82, change androgen receptor (AR) signaling and function. To accomplish the tasks outlined in our Statement of Work we fist generated stable cell lines of PC3 and DU145 cells which either had an empty vector introduced or CD82 cDNA. CD82 expression was detected by immunoblotting or immunofluorescence staining. During our studies we found it necessary to also generate DU145 cells over expressing c-Met or over expressing c-Met and CD82 together. We have also generated Adenoviruses that express an shRNA to CD82 and a cDNA for AR so we can study CD82 in normal cells. In addition, we have generated stable PC3 cell lines expressing CD9 and two CD82/CD9, and CD9/CD82 chimeric mutants, as well as several HA-tagged EC2 domain CD82 mutants. We have also generated a CD9-specific siRNA. Finally, we are developing both a transgenic mouse model and a conditional mouse deletion of CD82 to test the role of c-Met in CD82-mediated metastasis suppression *in vivo*.

Summary of Aim 1: The goal of Aim 1 in our Statement of Work was to examine the levels of $\alpha6\beta1$ and $\alpha3\beta1$ integrin expression in normal and tumorigenic prostate cells and monitor the effect of expression of CD82. The **first task** was to measure the levels of $\alpha6$, $\alpha3$ and $\beta1$ integrins by FACS in normal and tumor cell lines. We compared the expression of $\alpha2$, $\alpha3$, $\alpha5$, $\alpha6$, $\beta1$ and $\beta4$ integrins in primary prostate epithelial cells (PECs) with that of two tumor cell lines, PC3 and DU145. As shown in **Fig 1A**, PC3 cells had elevated levels of $\alpha2$, $\alpha6$, $\beta1$, and $\beta4$ compared to normal cells, while DU145 cells had elevated levels of $\alpha5$ and $\beta4$ integrin. In collaboration with Dr. Knudsen we also compared integrin expression in human normal and prostate cancer tissue sections by immunostaining. Staining of prostate cancer tissues indicates that tumors express predominantly $\alpha6$ and $\beta1$, have reduced levels of $\alpha2$, and have lost expression of $\alpha3$ and $\beta4$ as previously reported (**Fig 1B**) (1,2).

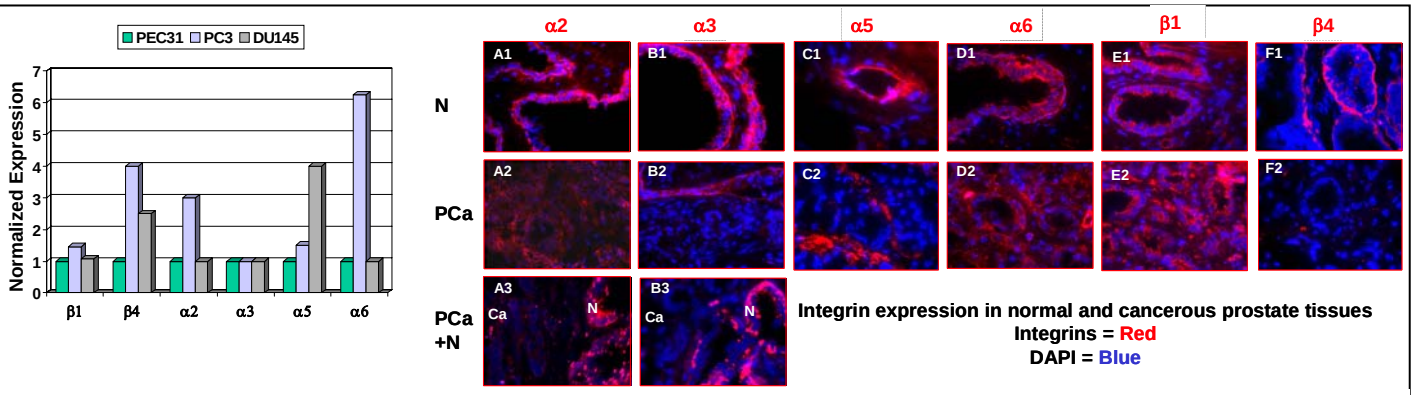


Fig. 1: A) Fold expression of integrins in PC3 and DU145 cells relative to PECs as determined by FACS. **B)** The normal (N) prostate gland consists of a continuous basal epithelial cell layer that adheres to the basement membrane, and a suprabasal luminal and secretory cell layer. Integrins α2, α3, α6, β1 and β4 are expressed in basal epithelial cells, while integrin α5 is expressed in endothelial cells (A1-F1). Integrins α2 and α3 levels are higher in basal than in secretory cells (A1, B1) while α6 and β4 are only expressed in basal cells (D1, F1). Expression of α2 and α6 integrins is observed in a large fraction of prostate cancer (PCa) cases (A1, D1), however the amount of α2 is less than in basal cells (A3). In our limited number of cancer cases (n=10), none expressed α3 (B2, B3) or β4 (F2). β1 integrin expression occurs equally in normal and prostate cancers (E2).

Our findings in the tumor cell lines differ considerably from those of the primary tumors in the tissue sections. There are several possible explanations. First, the tumor cell lines are well established lines that have been cultured for many years. Adaptation to growth in culture may force changes in expression that would not be observed in vivo. Second, PC3 and DU145 cells are derived from metastatic disease while the tissues we examined were primary tumors. Progression to metastasis may be accompanied by changes in integrin expression. However, if this is the case, the changes are not consistent.

The limitations of these studies are that we only looked at two tumor cell lines and we have not investigated integrin expression in metastatic tissues. However, these data do indicate that there are considerable differences in integrin expression levels in normal, primary, and metastatic tumors. It is interesting to note that the metastatic cells, while varying widely in their relative levels of expression, express the same wide repertoire of integrins as basal cells, while the primary tumors have a very restricted integrin profile, predominately α6 and β1 integrins. These studies do point out the possible validity of a recently proposed model that suggests that the cells in metastatic tumors may be derived from a more primitive or basal cell population compared to the cells in primary tumors (3,4). More thorough studies on integrin expression in metastatic prostate tissues are warranted to resolve these issues.

Loss of KAI1/CD82 expression could affect integrin expression. Therefore, our **second task** was to determine if re-expression of KAI1/CD82 in metastatic tumor cell lines affects the levels of integrin expression. Several clonal PC3 cell lines expressing CD82 were isolated and the levels of CD82 expression were measured by immunoblotting, FACS, and immunofluorescence staining (**Fig 2**).

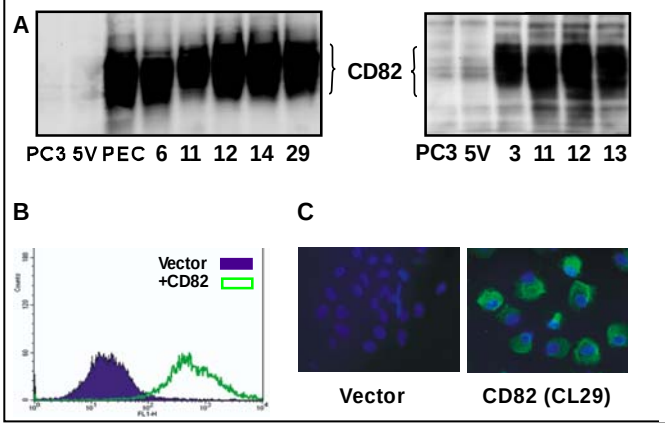


Fig 2: A) Several PC3 cell clones stably transfected with CD82 cDNA were analyzed by immunoblotting for CD82 expression. CD82 levels were compared to 5V cells which were transfected with vector alone and PECs; primary prostate epithelial cells expressing endogenous levels of CD82. **B)** Surface expression of CD82 was monitored by FACS. Data from one clone is presented. **C)** CD82 distribution (green) was monitored by immunofluorescence staining of vector and CD82 expressing cells. Cell nuclei were counterstained with DAPI (blue).

We used cell adhesion to laminin, surface labeling of α6 integrin, and FACS analysis to monitor the effects of CD82 expression on integrins. The ability of cells to adhere to increasing concentrations of laminin was not altered by CD82 expression (Fig 3A). The amount of α6β1 and α6β4 integrin on the cell surface was not altered by CD82 expression (Fig 3B,C). CD82 expression also had a negligible effect on α2, α3, α5, α6, β1, and β4 integrin surface expression as determined by FACS (not shown).

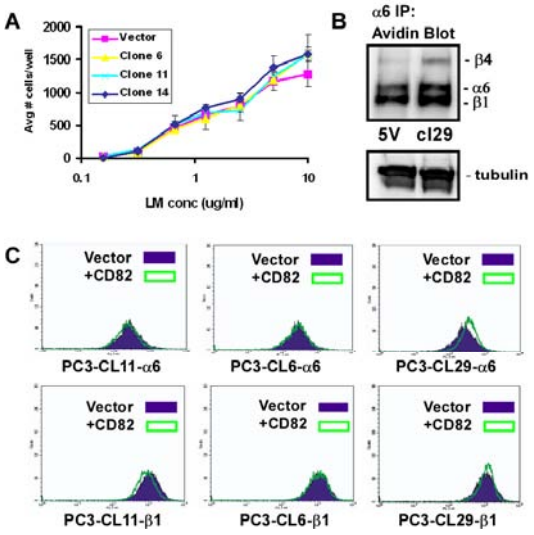


Fig 3: A) PC3 cells expressing CD82 (clone 6, 11, or 14) or vector were plated in triplicate on the increasing concentrations of laminin for 45 minutes. Non-adherent cells were washed away and stained. The number of adherent cells were counted in two random fields per well, averaged, and the standard deviation calculated. **B)** PC3 cells expressing CD82 (cl29) or vector (5V) growing in culture were labeled with biotin at 4°C. Integrin α6 was immunoprecipitated and the levels of biotin labeling were monitored by blotting with anti-avidin. Total levels of protein in the extracts were equal as indicated by immunoblotting for tubulin (tubuiln). Both β1 and β4 integrins were detected in the α6 immunoprecipitates. **C)** FACS analysis of cell surface expression of α6 and β1 integrins in vector and three CD82 expressing PC3 clones.

Our **third task** was to determine if loss of CD82 expression in normal cells alters the levels of α6β1 or α3β1 integrins. We generated an siRNA to specifically inhibit expression of CD82 in human cells. Transient transfection of CD82 siRNA into human primary prostate epithelial cells (PECs) for 72 hours was sufficient to inhibit CD82 expression by over 90% (**Fig 4**). The levels of CD82 do not change when cells are in suspension versus when plated on laminin. We also noted that adhesion of PECs to laminin was unaffected by loss of CD82 expression, suggesting that the levels of laminin integrins are not significantly altered in PECs lacking CD82.

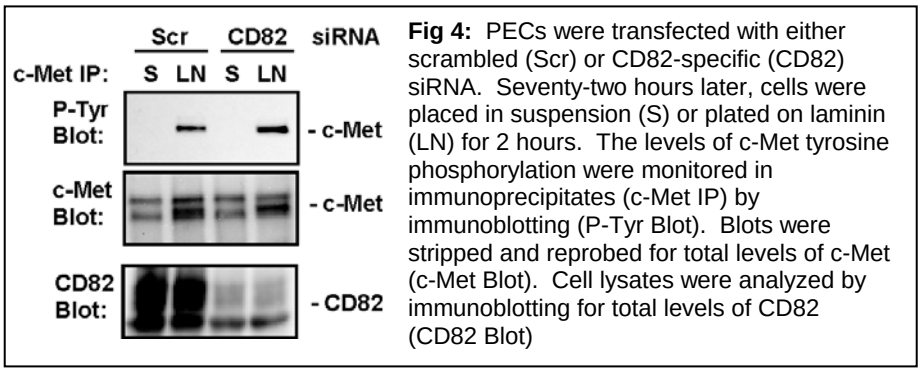


Fig 4: PECs were transfected with either scrambled (Scr) or CD82-specific (CD82) siRNA. Seventy-two hours later, cells were placed in suspension (S) or plated on laminin (LN) for 2 hours. The levels of c-Met tyrosine phosphorylation were monitored in immunoprecipitates (c-Met IP) by immunoblotting (P-Tyr Blot). Blots were stripped and reprobed for total levels of c-Met (c-Met Blot). Cell lysates were analyzed by immunoblotting for total levels of CD82 (CD82 Blot)

To date our data indicate that CD82 does not appear to function in the regulation of the cell surface expression of laminin integrins per se. This is in agreement with previous studies on other tetraspanin molecules known to associate with integrins (5,6). Tetraspanins appear to regulate integrin function, but not expression. However, a recent paper indicates that CD82 may regulate surface expression of α6 integrin in some cells (7). Additionally, CD82 does not appear to affect the adhesive functions of integrins. CD82 expression in our tumor cell lines did not alter the ability of the cells to adhere to any matrices. This is also in agreement with previous studies on tetraspanins (5,6). Together these data indicate that any alterations in integrin function due to CD82 expression are not due to changes in integrin expression or affinity for ligand and are most likely due to downstream signaling events initiated by integrins. Our findings, as outlined in the Summary of Aim 2 below, strongly support this observation.

Summary of Aim 2: The goal of Aim 2 was to determine how expression of CD82 affects the function of α6β1 and α3β1 integrins in normal and tumorigenic prostate cells. A significant portion of the findings in this section have been published (8). A copy of the reprint is attached. Our **first task** was to determine what the effect of changing CD82 expression has on cell migration. DU145 or PC3 cells stably transfected with vector or CD82 cDNA were tested for their ability to migrate towards three different matrices, collagen, laminin, or fibronectin. Both cell lines were able to adhere to all three matrices equally well at the concentration of matrix used, however, they clearly displayed differences in their ability to migrate. PC3 cells preferred to migrate on collagen or laminin, while DU145 cells preferred fibronectin (**Fig 5**). This directly correlated with the levels of

integrin expression seen by FACS and the respective ligands (see **Fig 1**). PC3 cells used their high levels of α2 and α6 integrins to migrate on collagen and laminin respectively, while DU145 used α5 integrins to migrate on fibronectin. Interestingly, CD82 suppressed migration of PC3 cells on both collagen and laminin, but failed to suppress migration of DU145 on fibronectin.

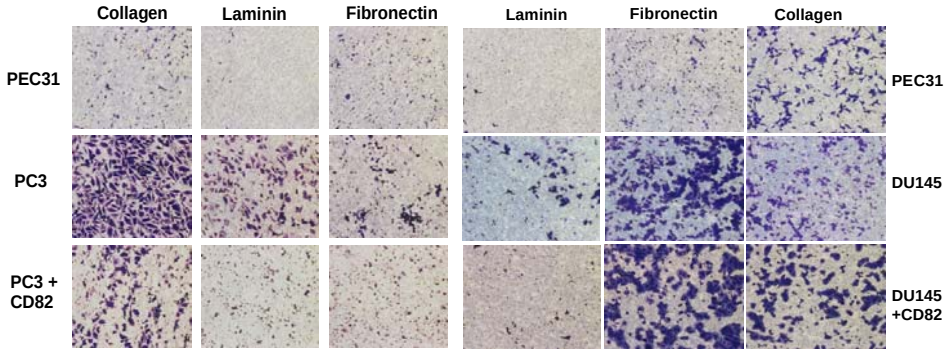
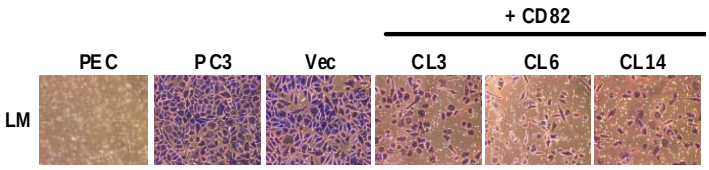


Fig 5: PC3 or DU145 cells were stably transfected with vector or CD82 cDNA. The ability of normal cells (PEC31) or the tumor cells to migrate towards collagen, fibronectin, or laminin in an 8μm Boyden chamber assay in the absence of growth factors or serum was monitored. Cells passing through the pores were stained with crystal violet and photographed.

These data support our initial hypothesis that CD82 regulates the function of laminin integrins. Combined with our results on integrin expression, these data further indicate that CD82 can have some effect on the collagen integrin, if it is sufficiently over-expressed. Our findings also indicate that CD82 has no effect on the expression of integrins or their ability to mediate adhesion, but rather functions to limit downstream effects of integrins.

Our **second task** was to determine how expression of CD82 effects integrin-mediated matrigel invasion. We have used the ability of cells to invade through matrigel and extrude through 8μm pored membranes coated with extracellular matrix as an *in vitro* measurement of invasiveness. PC3 and DU145 cells stably transfected with a vector or CD82 were tested for their invasive ability. PC3 cells, but not normal PECs were able to invade through matrigel towards laminin (**Fig 6**). All clones expressing CD82 demonstrated a dramatic reduction in invasive ability.

Fig 6: PC3 cell clones (CL#) stably transfected with vector or CD82, parental cells (PC3), or primary cells (PEC) were tested for their ability to invade through matrigel in an 8μm Boyden chamber to a laminin-coated (LM) membrane. Cells passing through the pores were stained with crystal violet and photographed.



When we tested the ability of CD82-expressing DU145 cells to invade matrigel, we found that invasion of normal DU145 cells through matrigel required the presence of the growth factor HGF (**Fig 7B**). HGF was not required for, nor did it enhance PC3 invasion (**Fig 7A**). The receptor for HGF is the receptor tyrosine kinase c-Met. We found that PC3 cells express significantly higher levels of c-Met than DU145 cells (see **Fig 10B**). We generated DU145 cells over expressing c-Met and these cells were now able to invade matrigel in the absence of HGF (**Fig 7B**) indicating that high levels of c-Met can overcome the need for ligand with respect to cell invasion of matrigel. Interestingly, expression of CD82 blocked invasion of DU145 cells mediated by eitherHGF or over expressed c-Met. However, at sufficiently high enough levels of HGF, the ability of CD82 to

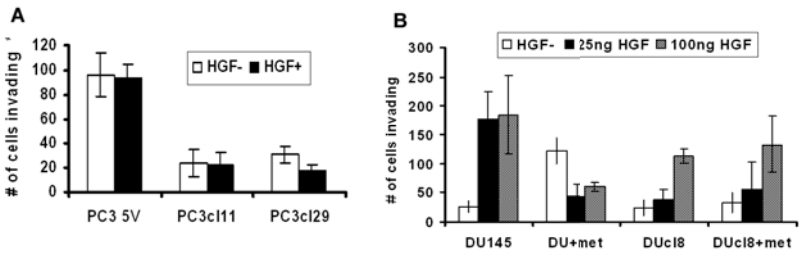


Fig 7: A) PC3 clones (cl11, cl29) stably transfected with CD82 or vector (5V) were tested for their ability to invade matrigel through 8μm Boyden chambers to a laminin coated membrane in the absence (HGF -) or presence of 25ng/ml HGF. **B)** Parental DU145 cells (DU145) or DU145 cells over expressing c-Met (DU+met), CD82 (DUcl8), or both c-Met and CD82 (DUcl8+met) were allowed to invade matrigel in the absence (HGF -) or presence of 25ng/ml or 100ng/ml HGF.

suppress invasion was abrogated. Thus CD82 negatively regulates both integrin and ligand-mediated invasion, and acts to limit c-Met activation rather than to completely suppress it. Therefore, we conclude that that function of CD82 is to control or limit the extent of c-Met activation. This conclusion is further supported by the data in **Fig 4**, where endogenous CD82 in normal prostate cells does not completely block c-Met activation, but its loss enhances c-Met activation.

Our **third task** in this aim was to characterize the role of CD82 in regulating the signaling events involved in migration and invasion. Using our PC3 clones expressing CD82 or vector transfected cells we monitored integrin-mediated activation of several different signaling pathways, focusing on those pathways thought to be involved in migration and invasion.

Previous studies had indicated that expression of CD82 in a human mammary cell line suppressed EGF-mediated activation of its receptor, EGFR (9). This was shown to be due to increased turnover of EGFR. Our laboratory has previously shown that integrins are capable of activating EGFR via a ligand-independent mechanism (10) and other laboratories have shown that other receptor tyrosine kinases, such as c-Met, PDGFR, and Ron can also be activated by integrins (11-13). c-Met, the receptor for HGF, has been implicated in promoting metastasis in many different types of tumors (14-16), including prostate cancer (17-23). The role of EGFR/ErbB2 in prostate cancer, unlike breast cancer, remains controversial (24-27). However, the role of c-Met in metastatic prostate cancer is more strongly supported (17-23). Thus, we investigated whether integrin signaling to c-Met was regulated by CD82. Adhesion of PC3 cells to three different matrices, laminin, collagen, or fibronectin, induced tyrosine phosphorylation of c-Met (**Fig 8A,B**). Expression of CD82 suppressed integrin-induced c-Met phosphorylation. This was observed in 6 different clones expressing CD82, but not in the vector-transfected cells. In PC3 cells EGFR was not significantly induced by integrins and its activity was unaffected by CD82 (**Fig 8C**). Treatment of PC3 cells with increasing concentrations of HGF stimulates c-Met activation. Expression of CD82 reduces the extent of c-Met activation by HGF (**Fig 8D**). Thus CD82 affects both matrix and ligand-mediated activation of c-Met. To further explore this idea, we inhibited CD82 expression in primary prostate epithelial cells using siRNA and found that loss of CD82 resulted in a two fold increase in matrix-induced activation of c-Met (see **Fig 4**).

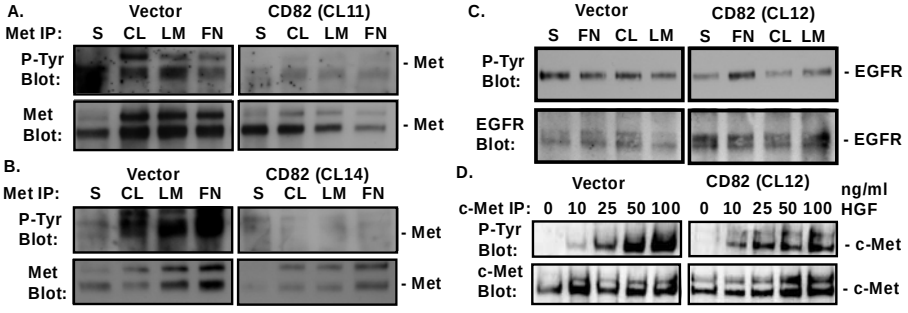


Fig. 8: PC3 cell clones stably transfected with vector or CD82 were placed in suspension (S) or plated on collagen (CL), laminin (LM), or fibronectin (FN). One hour later the level of **A,B)** c-Met or **C)** EGFR tyrosine phosphorylation was measured by immunoblotting of immunoprecipitates with anti-phosphotyrosine antibodies. **D)** PC3 cells plus or minus CD82 were treated with increasing concentrations of HGF and the levels of c-Met activation measured as above.

Signaling through Src has been proposed to be involved in regulating cell migration and invasion, and both integrins and c-Met are effective at activating Src (28-37). Therefore, we investigated whether expression of CD82 affects signaling to Src kinases. Adhesion of PC3 cells to matrices induced tyrosine phosphorylation of Src on the activation loop Y418 site, indicating that Src is activated (**Fig 9**). To further support these findings, we monitored the phosphorylation of Src substrates implicated in regulating cell

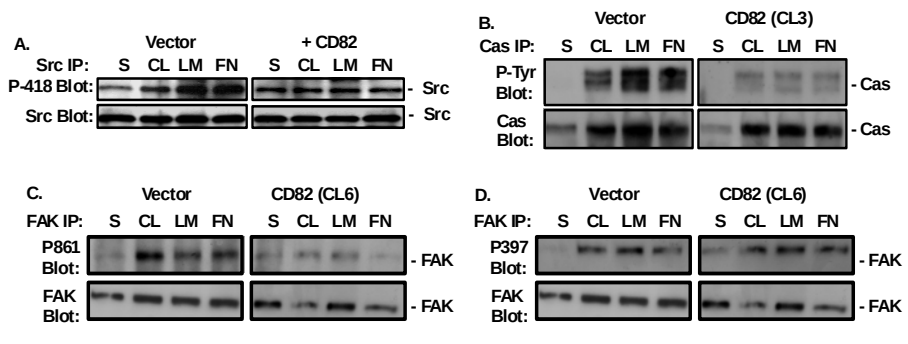


Fig 9: PC3 cell clones stably transfected with vector or CD82 were placed in suspension (S) or plated on collagen (CL), laminin (LM), or fibronectin (FN). One hour later the level of **A)** Src Y418, **B)** Cas, **C)** FAK Y861, or **D)** FAK Y397 phosphorylation was measured by immunoblotting of immunoprecipitates.

migration and invasion, namely FAK and p130Cas. Expression of CD82 inhibited tyrosine phosphorylation of p130Cas and reduced phosphorylation of FAK at Y861, a Src-specific site, but not at Y397 (**Fig 9B,C,D**). These results were observed with 4 different CD82-expressing clones. Thus CD82 expression affects integrin-mediated signaling to c-Met and to Src and its downstream substrates.

Given that CD82 can regulate the activity of Src and c-Met and various publications have suggested that c-Met can regulate Src and Src can regulate c-Met, we sought to determine if there was a relationship between c-Met and Src and their inhibition by CD82 (13,38). Inhibition of Src activity by the Src-specific inhibitor SU6656 (39), had no effect on integrin-mediated c-Met activation (not shown); however, it did block the ability of PC3 cells to invade matrigel (**Fig 10A**). To inhibit c-Met we designed an siRNA sequence to specifically block c-Met expression. Transfection of the c-Met siRNA failed to block integrin-induced activation of Src (not shown). However, c-Met shRNA expressing adenoviruses obtained from Dr. George Vande Woude (40) blocked invasion (**Fig 10B,C**). Thus, loss of CD82 expression in primary tumors enhances signaling independently through c-Met and Src, both of which are required for invasion.

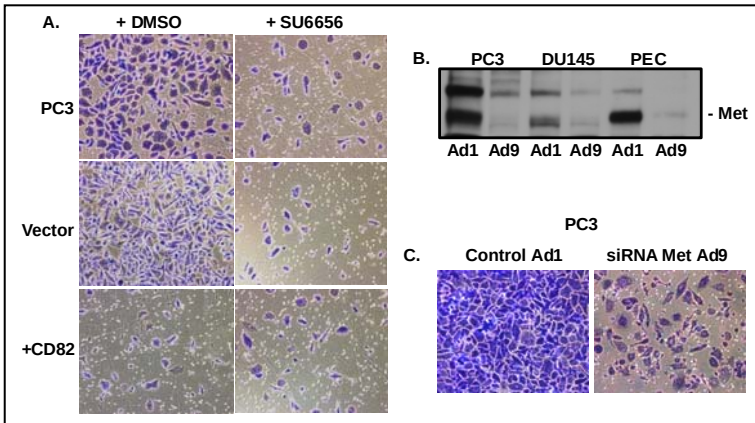


Fig 10: A) Parental, vector, or CD82 stably transfected PC3 cells were left untreated (DMSO) or treated with SU6656 and their ability to invade matrigel and migrate through 8μm pores was monitored. Cells passing through were stained with crystal violet and photographed. **B)** PC3, DU145 or PECs were infected with control Adenovirus (Ad1) or c-Met shRNA Adenovirus (Ad9). The levels of c-Met expression were measured by immunoblotting. **C)** c-Met shRNA Adenovirus-infected PC3 cells were tested for their ability to invade matrigel as outlined in **A)**.

The next question we wished to address is how CD82 suppress c-Met activation. Despite several attempts with different antibodies and lysis conditions we have been unable to detect an interaction between CD82 and c-Met either by coimmunoprecipitation or immunostaining (**Fig 11**). Thus either the interaction is very transient or there really isn't an interaction. Therefore we are proposing the CD82 regulates c-Met indirectly and have designed several approaches to investigate this.

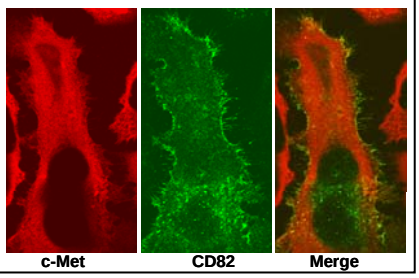


Figure 11: PC3 cells expressing CD82 were plated on laminin for 2 hours and stained with anti-c-Met (red) and anti-CD82 (green) antibodies. No overlap in staining was observed.

Expression of CD82 in a human breast cancer cell line was shown to increase the turn over of EGFR (9). It is possible that CD82 also regulates the turn over of c-Met. Therefore, we monitored the surface expression of c-Met in vector and CD82 expressing PC3 cells by FACS and by biotin-labeling. Under neither of these conditions did we see any change in the surface expression of c-Met when CD82 was over expressed (**Fig 12**). Even though c-Met activation decreased 4-6 hours after stimulation with matrix, there was no difference in the peak or when the loss occurred in CD82 expressing cells. We also failed to detect any significant labeling with ubiquitin (not shown). Thus CD82 does not alter the kinetics or turnover of c-Met after plating on matrix.

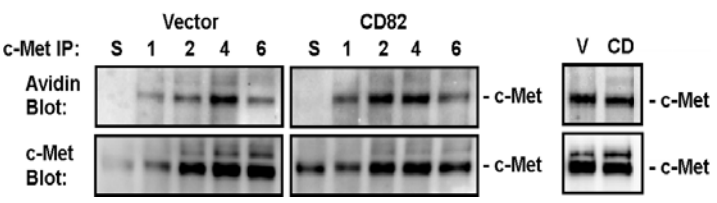


Fig 12: Control PC3 cells (Vector, V) or CD82 expressing PC3 cells (CD82, CD) were placed in suspension (S) or plated on laminin for **A)** 1, 2, 4, or 6 hours or **B)** for 4 hours. Cells were then labeled with biotin and c-Met was immunoprecipitated (c-Met IP). The levels of biotin labeling were monitored by immunoblotting with strepavidin (Avidin Blot). Blots were stripped and reprobed for total levels of c- Met (c-Met Blot).

CD82 is a member of the tetraspanin family. Tetraspanins are thought to act as "molecular facilitators" at the cell surface by organizing protein-protein complexes (41). Therefore, we sought to determine if CD82 might have an effect of the cell surface distribution of c-Met. Aggregation and cross phosphorylation of c-Met at the cell surface is required for its activation. Expression of CD82 in PC3 cells (**Fig 13A**) disrupts the c-Met aggregates found on the surface in the control cells. In DU145 cells CD82 suppresses the accumulation of c-Met aggregates in lamellipodia and c-Met is again diffusely distributed over the cell surface in small membrane protusions that look like blebs (**Fig 13B**). Thus CD82 alters the distribution of c-Met on the cell surface resulting in reduced aggregates of c-Met, and thereby limiting its ability to be activated.

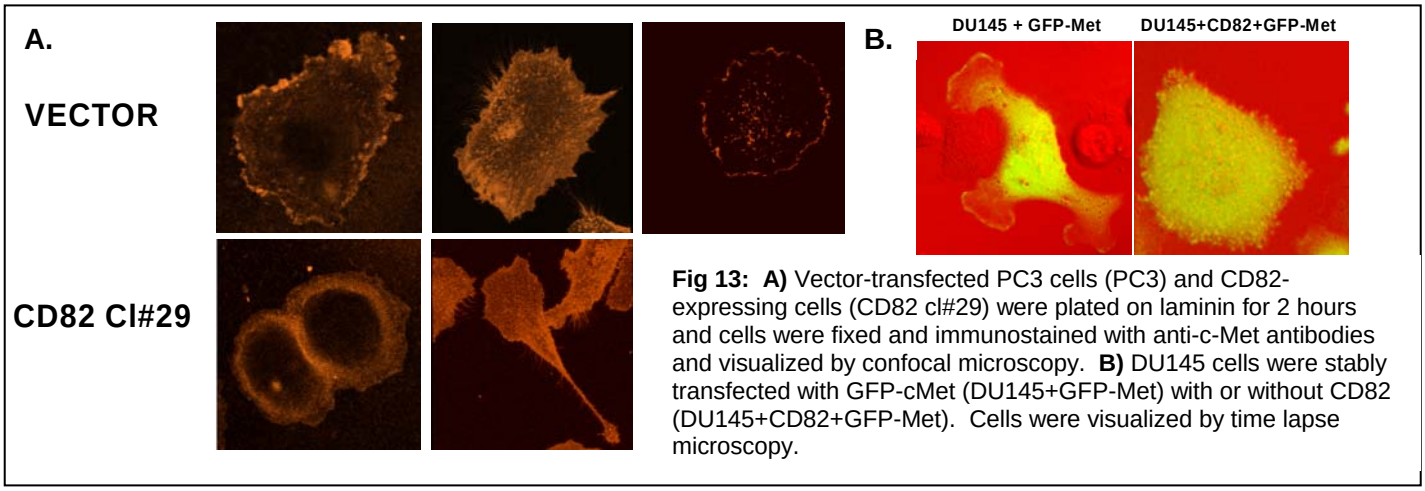


Fig 13: A) Vector-transfected PC3 cells (PC3) and CD82-expressing cells (CD82 cl#29) were plated on laminin for 2 hours and cells were fixed and immunostained with anti-c-Met antibodies and visualized by confocal microscopy. **B)** DU145 cells were stably transfected with GFP-cMet (DU145+GFP-Met) with or without CD82 (DU145+CD82+GFP-Met). Cells were visualized by time lapse microscopy.

We are using co-immunoprecipitation and mutagenesis approaches to identify which CD82-interacting molecules regulate c-Met activation. In the mutagenesis studies we are determining which domain on CD82 is responsible for suppressing c-Met activity. Our first approach was to use chimeric molecules of CD82 in which specific domains of CD82 are swapped with those of other tetraspanins. This approach has been used successfully in the past to identify functional tetraspanin domains (42,43). We chose to use chimeras between CD82 and CD9 because these chimeras already existed in the literature, and CD82 and CD9 are more distantly related to each other than the other commonly studied tetraspanins. Thus they would likely have distinct functions in the cell. We successfully generated stable cell lines in PC3 cells expressing full length CD9, and two different chimeras of CD82 and CD9 where the C-terminal and N-terminal halves of the molecules were interchanged. A preliminary screen using 4 different isolates expressing these CD9 molecules indicated that while CD9 itself was unable to suppress c-Met activation by integrins, either chimera still suppressed c-Met activation (**Fig 14**).

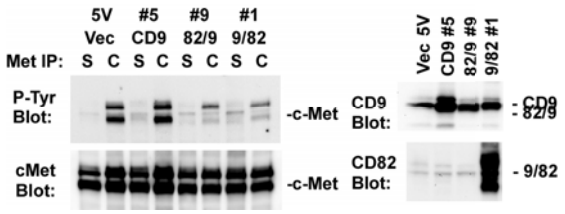


Fig 14: Stable PC3 clones expressing vector (Vec), CD9, CD9/82 or a CD82/9 chimera were placed in suspension or plated on collagen (C) and the level of c-Met activation was monitored by immunoblotting of c-Met immunoprecipitates (Met IP) with anti-phosphotyrosine antibodies (P-Tyr Blot). Total levels of c-Met in the IP were monitored by immunoblotting with anti-c-Met antibody (cMet Blot). Levels of expression of CD9 and each chimera were determined by immunoblotting of cell lysates with anti-CD9 or CD82 antibodies.

Another mutagenesis approach was to generate point and deletion mutations within the second extracellular domain (EC2) of CD82. Based on previous structure function analysis of tetraspanins, the EC2 domain is the most unique to each tetraspanin and is thought to be one of the primary determinants of tetraspanin-specific function (44,45). Thus if CD82 is specific to its effects on c-Met it is likely that this domain is important. This domain is also responsible for the ability of some tetraspanins to interact with integrins (46,47). Within the CD82 EC2 domain there are 6 cysteine residues that are proposed to generate three disulfide bonds (48). The middle disulfide bond in CD82 is the most divergent from other tetraspanins and likely to be important in its function. Therefore we generated point mutations of these two cysteine

residues, as well as a deletion mutant that would remove this section of the EC2 domain (**Fig 15**). An HA-tag was added at the C-terminus to allow us to monitor expression, as disruption of the EC2 domain will prevent CD82 antibody from binding.

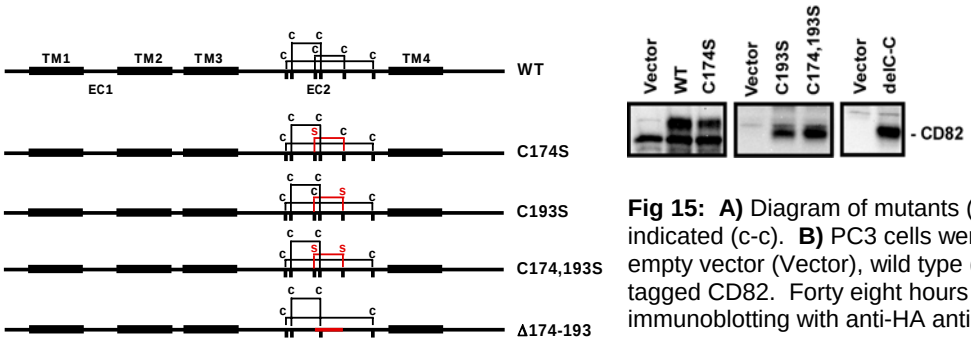


Fig 15: A) Diagram of mutants (red) generated with disulfide bonds indicated (c-c). **B)** PC3 cells were transiently transfected with 4ug of empty vector (Vector), wild type (Wt) and several EC2 mutants of HA-tagged CD82. Forty eight hours later cells were lysed and analyzed by immunoblotting with anti-HA antibody.

CD82 is known to interact with several other molecules, including integrins, other tetraspanins, and PKC. Therefore we tried immunoprecipitating CD82 and these molecules to determine if we can detect an interaction. So far we have not been able to detect an interaction between CD82 and PKC. However, we have detected an interaction between CD82 and the tetraspanin CD9 and β1 integrin (**Fig 16A**). These data, combined with the chimera data (see **Fig 14**) suggest that the ability of CD82 to interact with CD9 might be important for suppressing c-Met activation. We generated an siRNA to CD9 to determine if loss of CD9 prevents CD82 from suppressing c-Met (**Fig 16B**). Loss of CD9 prevented c-Met suppression by CD82 in response to HGF (**Fig 16C**). Thus the ability of CD82 to suppress c-Met may be mediated by interactions between integrins, CD82, and CD9.

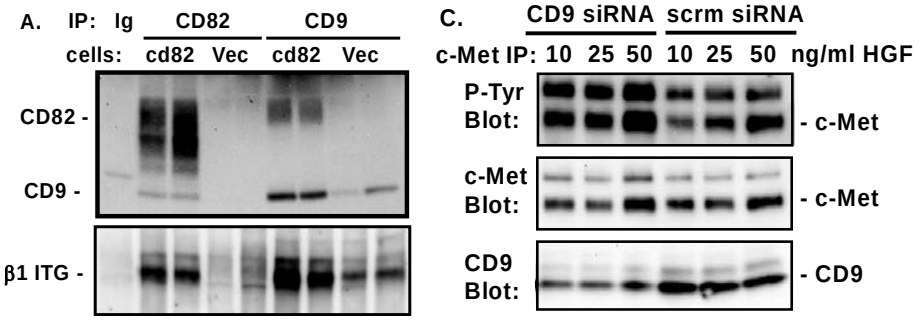


Fig 16: A) CD82 or CD9 were immunoprecipitated (IP) from adherent vector-transfected (Vec) or CD82 expressing (CD82) PC3 cells in duplicate. Immunoprecipitates (IP) were monitored for the presence of CD9, CD82, or β1 integrin (β1 ITG) by immunoblotting. Mouse immunoglobulin (Ig) was used as a negative IP control. **B)** PC3 cells were transiently transfected with scrambled (controls) or CD9 siRNA (siRNA) and the levels of CD9 monitored by immunoblotting. **C)** CD82-expressing PC3 cells were transiently transfected with scrambled (scrm) or CD9 siRNA and 72 hours later cells were stimulated with increasing concentrations of HGF and the level of c-Met activation was monitored by immunoblotting of c-Met immunoprecipitates with anti-phosphotyrosine antibodies (P-Tyr Blot). Total levels of c-Met in the immunoprecipitates and CD9 in cell extracts were monitored by immunoblotting (c-Met, CD9 Blot).

Upon activation, c-Met is tyrosine phosphorylated on several sites. CD82 expression reduces the overall tyrosine phosphorylation of c-Met, but does not completely abolish it. Thus, CD82 may target the loss of phosphorylation of only a subset of specific sites on c-Met. If so, this would suggest the potential regulation of a c-Met tyrosine phosphatase by CD82. Therefore, we have begun to analyze the level of tyrosine phosphorylation on the four known residues of c-Met. To date, we have detected a specific reduction in phosphorylation of Tyr1003 upon CD82 expression (**Fig 17**). Additional sites will be investigated and possible tyrosine phosphatases identified.

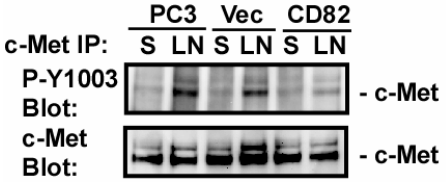


Fig 17: Parental PC3 cells (PC3), vector transfected (Vec), and CD82 expressing (CD82) cells were placed in suspension (S) or plated on laminin (LN) and the level of tyrosine phosphorylation on Y1003 was measured by immunoblotting of c-Met immunoprecipitates (c-Met IP) with site-specific phospho antibodies (P-Y1003 Blot). The blot was stripped and probed with anti-c-Met antibodies (c-Met Blot) to measure total levels of c-Met in the immunoprecipitates.

Summary of Aim 3: In Aim 3 we proposed to determine how androgen and integrins cooperate to regulate cell functions. We have written a solicited review article about the role of integrins and the androgen receptor (AR) in normal and prostate tumor cells (49). A copy is attached. In our studies we proposed to use primary prostate epithelial cells, since we had anticipated that primary cells would express the androgen receptor (AR). However, they do not (50). Therefore, we decided to re-introduce AR into both the primary and tumor cell lines. We generated retroviruses expressing wild type AR, infected PC3 cells, and selected several stable cell lines of both PC3 and DU145 expressing different levels of AR (**Fig 18**).

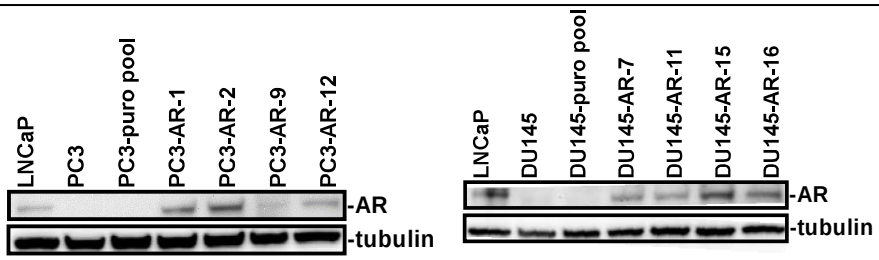
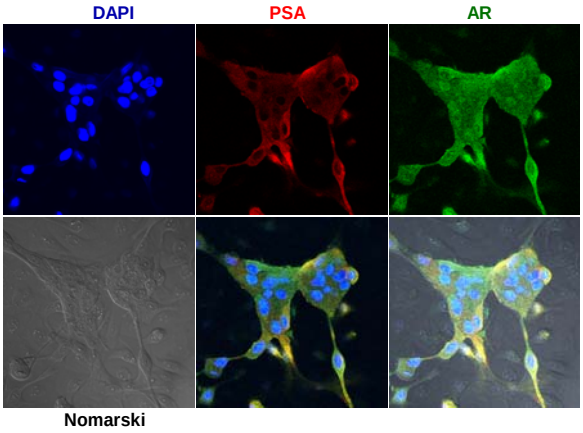


Fig 18: PC3 or DU145 cells were infected with retroviruses expressing wild type AR and stable cell lines were generated. Levels of AR expression were monitored by immunoblotting (AR). Expression was also monitored by immunofluorescence staining (not shown). LNCaP cells were used as a positive control for AR expression levels.

We are using several approaches to express AR in primary prostate epithelial cells (PECs). Since primary PECs do not infect well with retroviruses and we can not isolate stable lines we are using adenoviruses to express AR in primary PECs. However, we have recently been successful at generating two immortalized cell lines of PECs using E6/E7 and hTert. These cells are now past the 100 doublings stage. As far as we can tell they retain many of the characteristics of the primary cells. Thus we can try using our retroviruses to generate stable immortalized PEC lines expressing AR. In addition we can induce the expression of endogenous AR in primary PECs by culturing them under differentiation-inducing conditions. Treatment of confluent monolayers of PECs with KGF results in the induction of a double layer of cells and AR and PSA expression in the clusters of cells on the top layer (**Fig 19**). This structural differentiation resembles that observed *in vivo*, where AR expression is confined to the secretory cell layer that sits atop the basal layer. Thus we have generated several models in which we can study the interactions between AR and integrins, and ultimately determine whether CD82 alters their functions.



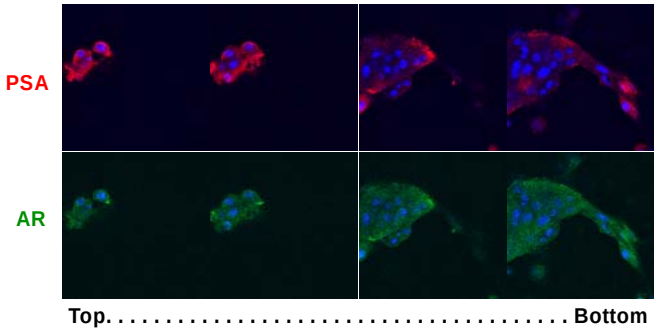


Fig 19: Primary PECs were plated on collagen and grown in the presence of KGF and DHT for 5-7 days. Cells were fixed and immunostained with anti-AR (green) or anti-PSA (red) antibodies and the nuclei counterstained with DAPI (blue). Cells were visualized by confocal microscopy. **A)** Only the clusters of cells on the top of the cell monolayer stained positive for AR or PSA. **B)** Sections through the clusters (Top to Bottom). AR expression was uniform through out the cells in the cluster, where as PSA was more highly concentrated at the top and edges exposed to the culture medium, as if it was actively being secreted. This suggests the cells are also polarized and secretory – analogous to what is seen *in vivo*. The differentiated cells also failed to express a majority of the integrins seen in basal cells, but retained some $\alpha 2\beta 1$ and $\alpha 6\beta 1$ integrins, which is the same as has been reported *in vivo* (not shown).

Our **first task** in Aim 3 was to determine if AR expression has an affect on cell migration. Expression of AR in PC3 cells dramatically altered their morphology when grown in culture. Normal PC3

cells are oval or slightly elongated in shape, while the AR-expressing cells were dramatically more spread with evidence of more lamellipodia and filapodia on the surface of the cells (**Fig 20**).

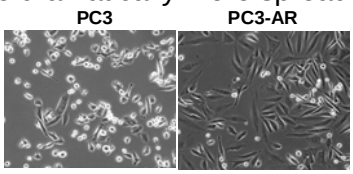


Fig. 20: PC3 cells infected with vector (PC3) or AR cDNA (PC3-AR) were grown under standard culture conditions. Phase microscopy images were taken at 10X magnification.

In preliminary studies in the PC3 AR expressing cells we noted several differences in the expression of integrins. Specifically we observed a decrease in α3, α5, β1, and β4 integrin in the PC3 AR expressing cells (**Fig 21**), leaving α2 and α6 integrins as the only integrins in these cells. Interestingly these integrins are exactly the same as those seen in primary tumor cells which express AR (see **Fig 1**; (51)). In addition, these same two integrins, (α2β1 and α6β1) are also the only integrins expressed in the normal cells when induced to differentiate with KGF (as in **Fig 19**; not shown). Thus AR appears to control integrin expression in both normal and tumor cells. We have not yet determined if the effects of AR on integrin expression are responsible for changes in migration or invasion on the different matrices.

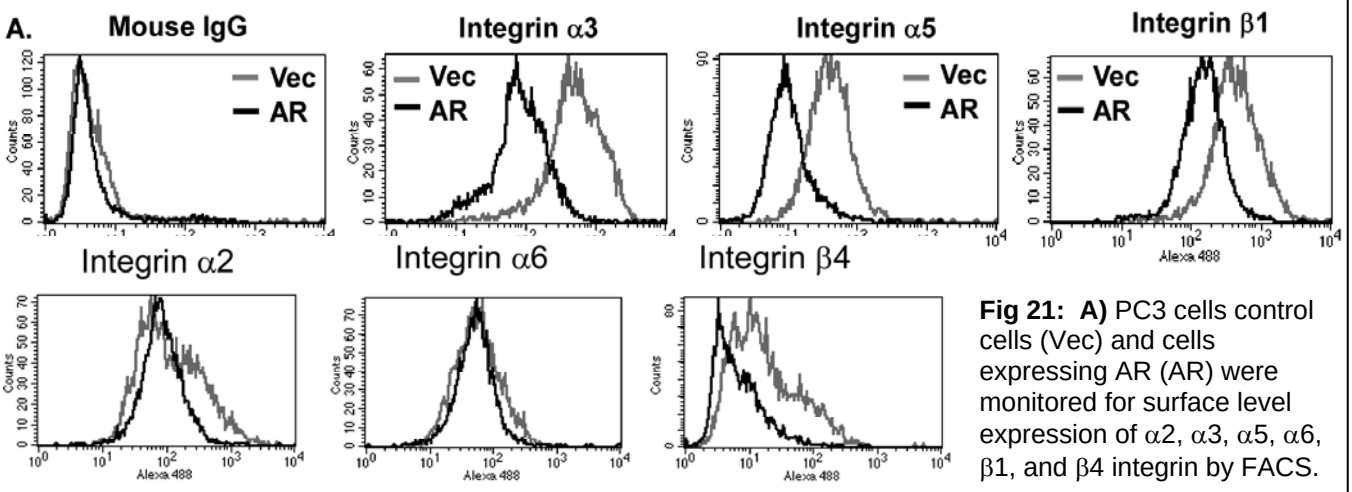
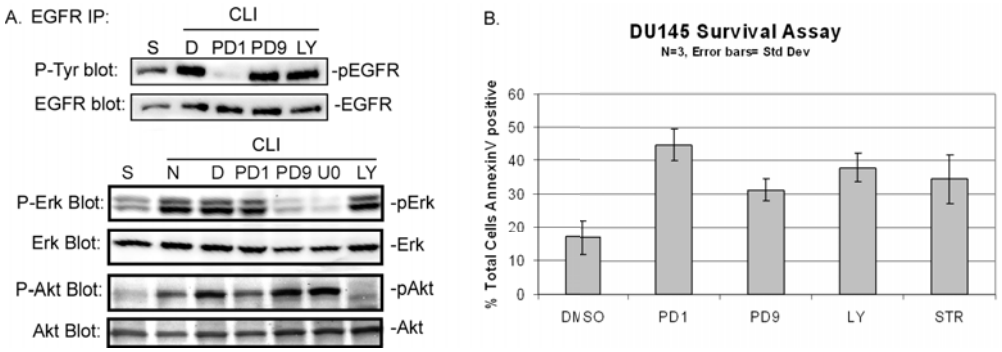


Fig 21: A) PC3 cells control cells (Vec) and cells expressing AR (AR) were monitored for surface level expression of α2, α3, α5, α6, β1, and β4 integrin by FACS.

Our **second task** was to determine if engagement of laminin integrins or re-expression of CD82 alters AR-dependent transcription or cell survival. We first determined the primary integrin-mediated cell survival pathways that are operating in both primary cells and in PC3 cells. Survival of primary prostate epithelial cells on laminin 5 (their endogenous matrix) is mediated by signaling through EGFR/Erk and Src. The PI-3K/Akt pathway was not activated on this matrix, and thus survival was not mediated by this pathway. In contrast, adhesion of PC3 cells to laminin failed to activate EGFR/Erk, and therefore this pathway was not important for integrin-mediated survival. On the other hand, inhibition of the PI-3K/Akt pathway or the Src pathway induced cell death in PC3 cells. These findings have recently been published and the reprint is attached (52). We have gone on to determine if these same survival pathways are important in the other prostate cancer cell line, DU145. Signaling through EGFR/Erk and PI-3K (**Fig 22**), but not Src (not shown) appears to be important for the survival of DU145 cells.

Fig 22: DU145 cells were plated on collagen (CL1) in the absence (D,N) or presence of PD168393 (EGFR inhibitor), PD98059 (MEK inhibitor), U0126(MEK inhibitor), LY294002 (PI-3K inhibitor), or staurosporine (STR). **A)** Cells were lysed and the level of EGFR, Erk, and Akt activation were monitored. **B)** 72 hours later the level of Annexin V positivity was monitored by FACS.



Knowing that signaling through PI-3K/Akt is important for survival of both PC3 and DU145 cells, we next determined whether AR would affect PI-3K/Akt-dependent survival. We plated vector-transfected PC3 cells or PC3-AR cells on laminin in the presence or absence of a PI-3K inhibitor and monitored their survival as measured by TUNEL and PI staining. Inhibition of PI-3K, dramatically reduced cell number and increased TUNEL staining and SubG1 content after 72 hours of culturing on laminin (**Fig 23A,B**). Expression of AR blocked the cell death on laminin induced by inhibition of PI-3K. Thus expression of AR is able to overcome the need for signaling through PI-3K/Akt to regulate survival on laminin. One potential explanation for the ability of AR to rescue cells treated with LY294002 is that in these cells Bad, a proapoptotic regulator, is not induced when AR is present and the total level of the anti-apoptotic protein, Bcl-XL, is dramatically increased in AR expressing cells (**Fig 23C,D**). Interestingly, the ability of AR to rescue survival in PI-3K inhibited cells did not require DHT, suggesting an androgen-independent mechanism.

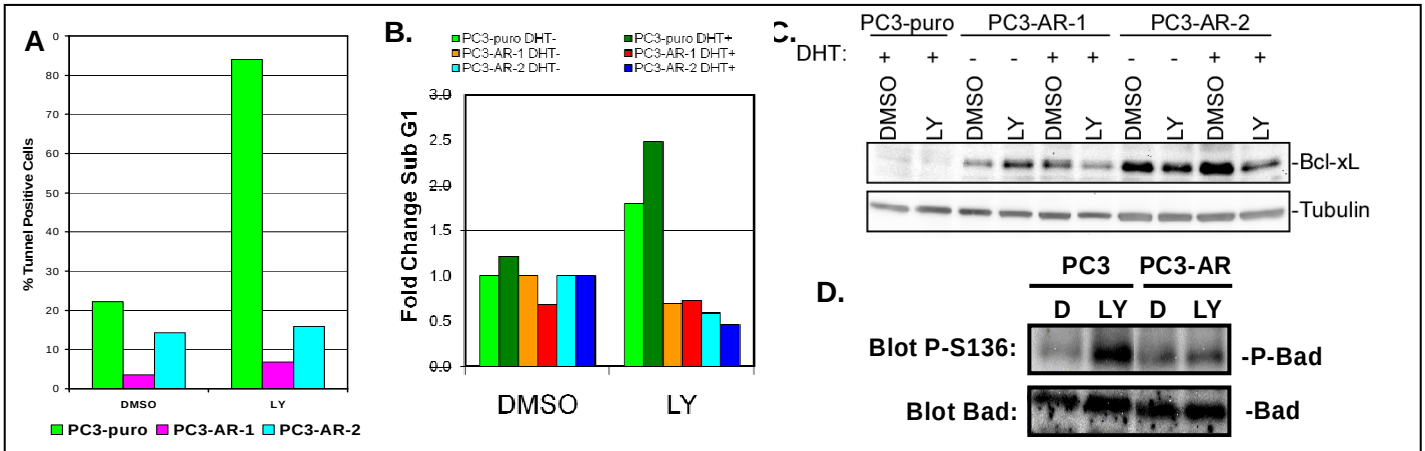


Fig 23: PC3 cells infected with vector (PC3-puro) or AR cDNA (PC3-AR-1,-2) were plated on laminin in the absence (DMSO) or presence of the PI-3K inhibitor (LY294002) plus or minus DHT. 72 hours later the cells were stained for **A)** DNA fragmentation using TUNEL or **B)** PI for DNA content and analyzed by FACS. Under these same conditions (D=DMSO, LY=LY294002), the levels of **C)** Bcl-XL or **D)** Bad phosphorylation on Ser136 (Blot P-S136) were monitored by immunoblotting.

Summary of Additional Work: One measure of success is the additional questions that the initial studies generated, that if answered will help further our understanding. The following results have also been generated and address additional important issues about the function of CD82. The data generated from these studies (as well as those above) were used as preliminary data for submission of additional grant applications.

CD82 was originally described as a metastasis suppressor gene, i.e. it does not affect tumor growth, but rather alters invasion and metastasis. This conclusion was based on subcutaneous injections as well as tail vein metastasis assays of a rat tumor cell line and a human mammary tumor cell line (53,54). However, one report indicated that while injection of a metastatic mammary cell line re-expressing CD82 did not alter the tumor incidence, it did result in smaller tumors (53). Since none of these assays had looked at human prostate cells, we decided to test the effect of expressing CD82 in our two prostate tumor cell lines on *in vivo* metastasis. To generate a more "realistic" metastatic model for prostate cancer we chose to use orthotopic injection of the tumor cells directly into the prostate gland. This method has previously been used to successfully generate lung metastases from PC3 cells (55,56). Wild type PC3 cells generated primary tumors in 72% of the mice (13/18), 69% of which metastasized to the lymph nodes (9/13) and 46% to the lungs (6/13). In contrast, no lung metastases were seen in the CD82 expressing cells and only 2 of 5 (40%) mice developed lymph-node metastases. Surprisingly, only 25% (5/20) mice injected with CD82 expressing cells generated tumors.

The tumors that were generated by the CD82 expressing cells were of the same size as those produced by the normal PC3 cells (**Fig 24A**). The few metastases and tumors that were observed in the

CD82-expressing tumor cells demonstrated a partial loss of CD82 expression, since some of the cells within these tumors and lymph node metastases no longer expressed CD82 (**Fig 24B**).

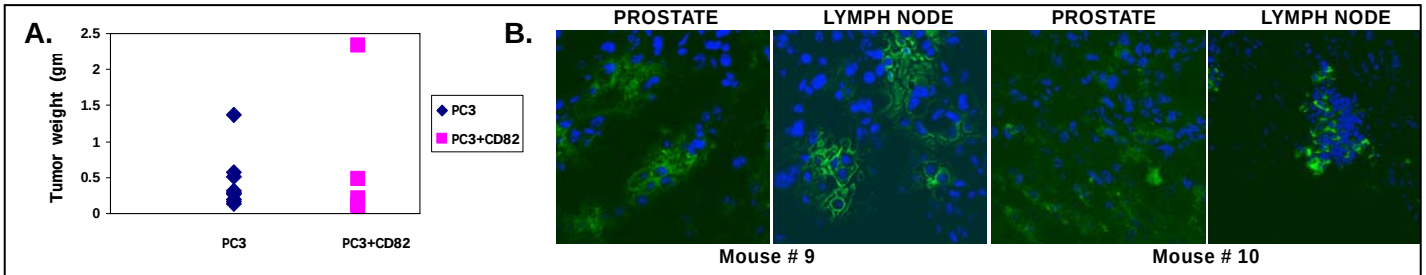


Fig 24: A) The prostates of nude mice were injected with normal PC3 cells (PC3) or PC3 cells expressing CD82 (PC3+CD82). Ten weeks later prostates were removed and tumors isolated. Tumors from each mouse were weighed. **B)** Immunofluorescence staining of mouse prostate tumors and lymph node metastases generated from CD82-expressing PC3 cells in nude mice. Frozen tissues in OTC were sectioned and immunostained with anti-human CD82 antibody (green) and nuclei were counterstained with DAPI (blue).

Thus these data indicate that CD82 expression not only suppresses metastasis, but also suppresses cell growth *in vivo*. We directly measured cell proliferation of normal and CD82-expressing PC3 cells and detected no differences in their proliferative potential *in vitro* (**Fig 25**). Thus something in the host environment is limiting tumor proliferation that is not present *in vitro.*

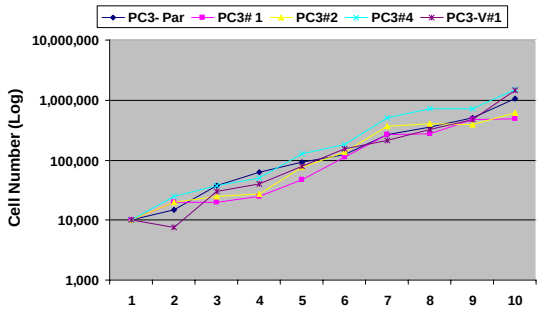


Fig 25: 10,000 parental PC3 cells (PC3-Par), vector-transfected cells (PC3-V#1), and three CD82 expressing clones (PC3#1, 2 and 4) were plated on day 1 and counted daily for 10 days.

Our model predicts that CD82 acts to suppress metastasis by inhibiting signaling through c-Met. We wish to test this hypothesis *in vivo* in a metastasis model. DU145 cells, when injected subcutaneously into SCID mice generate tumors, but when injected into a transgenic SCID mice over expressing human HGF, generate larger tumors (57). We wanted to determine if expression of CD82 would suppress HGF-induced increase in tumorigenesis or metastasis. Since we did not know if orthotopic injection of DU145 cells in these mice would lead to metastases we set up an initial trial. We orthotopically injected the prostates of SCID mice or HGF/SCID mice with the DU145 tumor cells and monitored the development of metastases. Interestingly, orthotopic injection of DU145 cells into the SCID or HGF/SCID mice resulted in 31 of 36 (87%) mice developing prostate tumors. However metastasis was observed only in the HGF/SCID mice and not the SCID mice;18/31 mice (58%) developed metastases. Thus the presence of HGF is required for the development of metastases by DU145 cells. We are now testing whether expression of CD82 in DU145 cells suppresses this HGF-specific induction of metastasis.

Several different prostate tumor mouse models have been developed that recapitulate prostate tumor development in humans. Loss of p27Kip (a cell cycle regulatory protein) and Pten (a negative regulator of the PI-3K pathway), and increased levels of c-Myc have all been demonstrated to occur in human prostate cancer. Correspondingly, loss of p27kip expression combined with partial loss of Pten and/or Nkx3.1, complete loss of Pten alone, or over expression of c-Myc can each lead to the development of prostate adenocarcinomas in mice (58-60). However, in these models, the development of metastases is rare and tumors do not metastasize to the bone or other organs as is observed in human disease. Our hypothesis is that genetic loss of CD82 in the context of primary prostate cancer is critical for the development of metastatic disease. Our approach is to first develop a CD82 conditional null mouse in which loss of CD82 expression is induced in the prostate. These mice will then be bred to one or more of the mouse models described above that predispose them to the development of primary prostate cancer. This model will allow us to determine if loss of CD82 in the context of primary prostate cancer is required for the development of prostate metastasis in mice.

Our strategy was to generate conditional loss of CD82 expression in prostate epithelial cells of the mouse. We generated a targeting vector for recombination in EX cells in which exons IV and V are flanked by LoxP sites (**Fig 26**). This targeting vector contains a neomycin resistance gene for positive selection and an HSV-TK gene for negative selection.

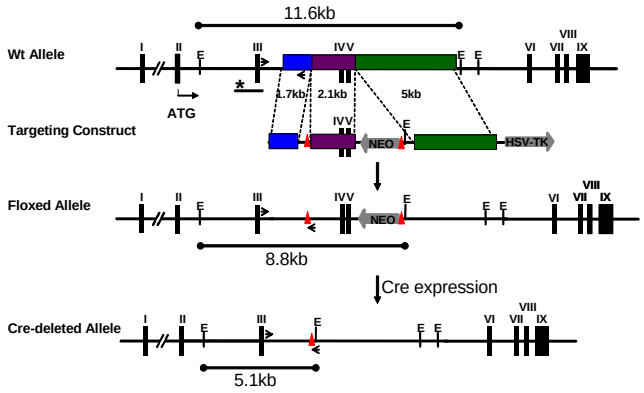


Fig 26: Targeting strategy for disruption of murine *cd82*. A BAC clone containing murine *cd82* was identified by screening of BAC library RPCI-22 (129/SvEvTACC/Br). Three PCR fragments of *cd82* were assembled into the targeting vector. Prior to assembly PCR fragments were subcloned and their sequences verified. A 2.1kb PCR fragment including a loxP site (red triangle) and exons IV and V was inserted in front of the neo gene. A 1.7kb PCR fragment immediately 5' of fragment 1 was inserted upstream of the loxP site (red triangle). A third 4kb PCR fragment beginning immediately 3' of fragment 1 was inserted downstream of the neo gene and a loxP site. ES cell clones were screened by PCR amplification of a region of *cd82* beginning in Exon III and terminating in the 5' most loxP site for the floxed allele (arrow heads). All PCR positive clones will be verified by Southern blotting with a ~0.5 KB probe (*****) close to, but not within, the region covered by the targeting construct. Digestion of genomic DNA with EcoRI combined with our Southern blotting probe will easily distinguish between Wt (11.6kb), floxed (8.8kb), and Cre-deleted alleles (5.1kb).

We obtained over 10 positive ES cell clones in which the floxed allele recombined correctly into the CD82 locus (**Fig 27A**). Two clones were selected for injection and 4 male chimeric mice with mixed coat color (agouti) were obtained. Two mice successfully generated agouti offspring, 20 of which we have confirmed to be heterozygous for the floxed CD82 allele (**Fig 27B**). The heterozygotes were bred to homozygosity (**Fig 27C**).

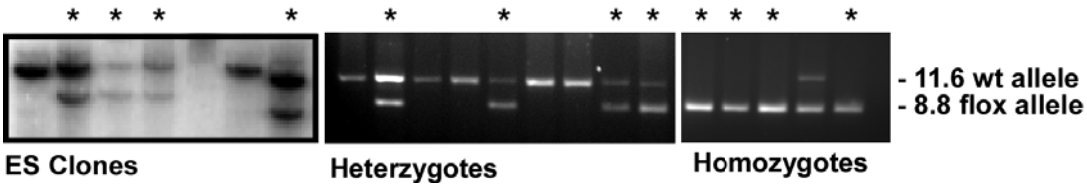
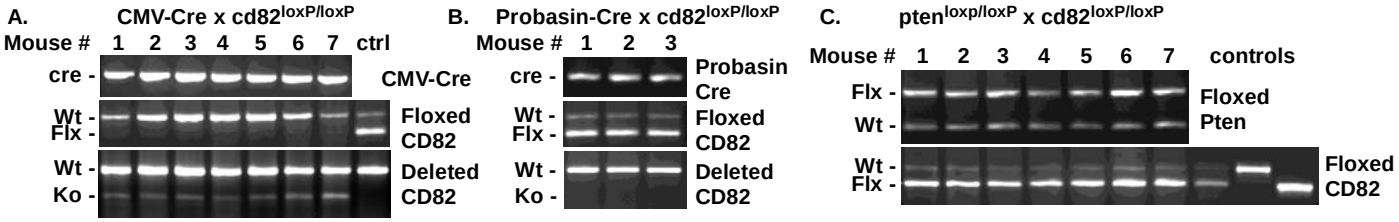


Fig 27: A) ES cells were screened for recombination of the floxed CD82 allele by Southern blotting. Examples of 4 positive clones are shown. **B)** Agouti F1 offspring from the chimeras were screened by PCR for the floxed CD82 allele. Four positive heterozygotes are shown. **C)** Offspring from the heterzyous cross were screened by PCR for double floxed alleles. 4 positives are shown.

We crossed homozygous CD82 floxed mice into CMV-Cre to verify generation of Cre-dependent loss of the floxed CD82 alleles (**Fig 28A**). In the heterozygotes from the CMV-Cre offspring one of the CD82 alleles is no longer recognized by the floxed allele PCR primers, but is now recognized by the deleted allele primers. The Cre/cd82$^{LoxP/+}$ mice were crossed to generate homozygous deletion of floxed CD82 (**Fig 29A**). Now both alleles are deleted. At 5 weeks of age, these mice appear normal and viable. Thus loss of exons IV and V in the CD82 gene in all tissues does not result in embryonic lethality.

The homozygous CD82 floxed mice were also bred to probasin-Cre mice and into the floxed Pten mice (58). We have mice from these crosses containing Cre positive heterozygous floxed CD82 alleles and double heterozygous floxed CD82 and Pten alleles (**Fig 28 B,C**). The Cre-deleted allele would not be expected to appear in the tail DNA of the probasin-Cre mice, since Cre is only active in the prostate. We have also now generated homozygous floxed cd82/Pten mice (**Fig 29B**). Probasin-Cre mice will be crossed with the double floxed homozygous CD82/Pten mice to determine if combined loss of CD82 and Pten in the prostate leads to the development of metastatic prostate cancer.

Figure 28: A) DNA isolated from CMV-Cre x cd82$^{loxP/loxP}$ and **B)** cd82$^{loxP/loxP}$ x Probasin-Cre offspring were screened by multi-plex PCR for the Cre (cre) gene and the floxed (Flx) or deleted (Ko) CD82 alleles. **C)** DNA isolated from cd82$^{loxP/loxP}$ x pten$^{loxP/loxP}$ offspring were screened by PCR for floxed (Flx) CD82 and Pten alleles.



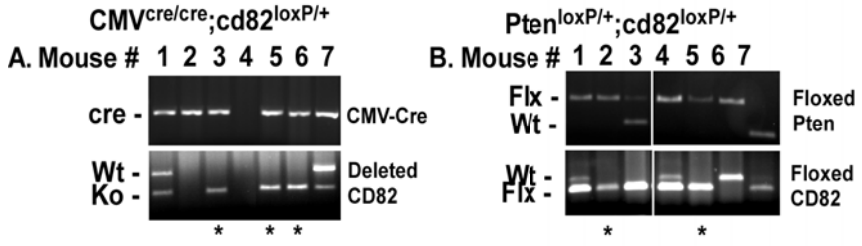


Fig 29: A) CMV-Cre;cd82$^{loxP/+}$ mice were crossed to each other to generate mice expressing Cre in all tissues which resulted in homozygous deletion of both the CD82 alleles as detected by multiplex PCR of tail DNA. **B)** pten$^{loxP/+}$;cd82$^{loxP/+}$ mice were crossed to each other to generate double homozygous pten$^{loxP/loxP}$;cd82$^{loxP/loxP}$ mice as determined by multiplex PCR of tail DNA. Astericks indicate mice of the desired genotype.

KEY RESEARCH ACCOMPLISHMENTS

In the first year:

1. Generated stable transfectants of PC3 and DU145 cells expressing CD82.
2. Demonstrated that CD82 expression does not affect laminin or collagen integrin expression.
3. Demonstrated that CD82 expression suppresses laminin and collagen-mediated migration.
4. Demonstrated that CD82 suppresses matrigel invasion.
5. Identified c-Met and Src as independent downstream targets of CD82 in PC3 cells.
6. Demonstrated that both Met and Src are required for PC3 cell matrigel invasion.
7. Observed that expression of androgen receptor in PC3 cells increases integrin-mediated cell survival when PI-3K is inhibited.

In the second year:

1. Demonstrated that CD82 also suppresses HGF-mediated activation of c-Met and HGF-mediated invasion.
2. Determined that CD82 modulates the activity of c-Met by decreasing its sensitivity to ligand.
3. Demonstrated that CD82 indirectly regulates c-Met activity by physically altering c-Met distribution on the cell surface, but not altering its degradation or internalization.
4. Generated adenoviruses for expression of CD82 shRNA and AR in primary prostate epithelial cells
5. Showed that loss of CD82 expression in primary cells enhances c-Met activation on laminin.
6. Developed an orthotopic metastatic mouse model for measuring the effect of CD82 expression on tumor growth and metastasis.
7. Determined that CD82 acts to suppress tumor growth *in vivo*, but not *in vitro*.
8. Identified the signaling pathways required for survival of normal versus metastatic prostate cells on laminin.

In the third year:

1. Generated stable PC3 cell lines expressing CD9, CD82/CD9 and CD9/CD82 chimeric mutants.
2. Generated HA-tagged CD82 and several HA-tagged EC2 domain mutants.
3. Determined that two regions of CD82, the EC2 domain and the N-terminus, are required for efficient inhibition of c-Met activation.
4. Demonstrated that re-expression of CD82 in PC3 cells induces the formation of a complex between CD82, β1 integrin, and another tetraspanin, CD9.
5. Demonstrated that CD9 is required for CD82-mediated suppression of c-Met activation in response to HGF
6. Demonstrated that CD82 specifically inhibits c-Met tyrosine phosphorylation at Y1003.
7. Developed an in vitro differentiation model of primary prostate epithelial cells resulting in induction and expression of AR and PSA in primary epithelial cells
8. Identified the integrin-mediated survival pathways in DU145 cells
9. Demonstrated that re-expression of AR in prostate tumor cells suppresses integrin expression and increases the levels of Bcl-XL expression independent of androgen.
10. Developed a transgenic mouse model to test the role of c-Met in CD82-mediated metastasis suppression.
11. Generated heterozygous and homozygous floxed alleles of the CD82 gene in mice.

REPORTABLE OUTCOMES

The following items have been generated due to the research carried out in the three years of funding.

1. We have published several papers resulting from this work. We anticipate another paper going out soon once we test the CD82 mutants as well as future manuscripts based on some of the initial data generated here.

Sridhar, S.C. and Miranti, C.K. 2005. "Tumor Metastasis Suppressor KAI1/CD82 is a Tetraspanin" in **Contemporary Cancer Research: Metastasis**. Eds. C. Rinker-Schaeffer, M. Sokoloff and D. Yamada. In press.

Sridhar, S.C. and Miranti, C.K. 2006. Tetraspanin KAI1/CD82 suppresses invasion by inhibiting integrin-dependent crosstalk with c-Met receptor and Src kinases. **Oncogene**, 25:2367–78.

Knudsen, B.S. and Miranti, C.K. 2006. The impact of cell adhesion changes on proliferation and survival during prostate cancer development and progression. **J. Cell. Biochem**., 99:345–361.

Edick, M.J., Tesfay, L., Lamb, L.E., Knudsen, B.S., and Miranti, C.K. 2007. Inhibition of Integrin-Mediated Crosstalk with EGFR/Erk or Src Signaling Pathways in Autophagic Prostate Epithelial Cells Induces Caspase-Independent Death. **Mol. Biol. Cel**l., 18:2481–2490.

2. Our work on CD82 was presented orally at several different meetings in the past three years.

Presented by Dr. Sridhar:

Sridhar, S.C, Knudsen, B.S., and Miranti, C.K. 2004. Role of CD82 in integrin function in prostate cell lines. **FASEB Summer Research Conference: "Advances in Tetraspanin Research",** Pine Mountain, GA, June 18-24.

Presented by Dr. Miranti:

Chinnaswamy, S., Knudsen, B. and **Miranti, C.K.** 2004. Assessing the role of CD82 loss in prostate tumor metastasis. **4th Annual Michigan Prostate Research Colloquium: "Basic and Clinical Advances in Prostate Cancer Research"**, Grand Rapids, MI, May 1.

Sridhar, S.C, Knudsen, B.S., and Miranti, C.K. 2004. Metastasis Suppressor CD82 Regulates Integrin-Mediated Signaling in Primary and Prostate Tumor Cells. **AACR: Basic, Translational, and Clinical Advances in Prostate Cancer.** Bonita Springs, FL, Nov 17-21.

Miranti, C.K. 2005. CD82 suppresses integrin-dependent crosstalk with c-Met to inhibit invasion. **Karmanos Breast Cancer Research Retreat**, Detroit, MI, November 11.

Miranti, C.K. 2006. CD82 and suppression of prostate metastasis. **6th Annual Michigan Prostate Research Colloquium:** University of Michigan, Ann Arbor, MI, May 6.

Miranti, C.K. 2006. CD82 Suppresses tumor Cell Invasion and c-Met Activation by Altering the Distribution of c-Met on the Cell Surface. **FASEB: Membrane Organization by Tetraspanins and Small Multi-transmembrane Proteins**. Tucson, AZ, July 22-27.

Miranti, C.K. 2006. Autophagy-Mediated Survival of Prostate Epithelial Cells Requires Crosstalk Between Integrins and Receptor Tyrosine Kinases. **14th ACS Great Lakes Cancer Symposium: Celebrating 60 Years of Cancer Research**. Grand Rapids, MI, Oct 16.

Miranti, C.K. 2007. Inhibition of Integrin-Mediated Crosstalk with EGFR/Erk or Src Signaling Pathways in Autophagic Prostate Epithelial Cells Induces Caspase-Independent Death. **Keystone: Autophagy in Death and Disease.** Monterey. CA. April 15-20.

Miranti, C.K. 2007. Killing Prostate Epithelial Cells. **7TH Annual Michigan Prostate Research Colloquium: "Basic and Clinical Advances in Prostate Cancer Research"**. Detroit, MI, May 12.

3. Data were presented at several scientific meetings as poster presentations.

Presented by Dr. Sridhar:

Sridhar, S., Repair, N.R., Ilian, B.S., Knudsen, B.S. and Miranti, C.K. 2005. Prostate tumor suppressor protein KAI1/CD82 suppresses invasion by inhibiting integrin-dependent crosstalk with c-Met receptor and Src kinases. **5th Annual Symposium Michigan Prostate Research Colloquium Basic and Clinical Advances in Prostate Cancer Research.** Wayne State University School of Medicine, Detroit, MI, April 23.

Suganthi Sridhar, Sharon Moshkovitz, Gary Rajah, Ilan Tsarfaty, and Cindy Miranti. 2006. The Tetraspanin CD82 Suppresses Tumor Cell Invasion and c-Met Activation by Altering the Distribution of c-Met on the Cell Surface. **Van Andel Research Institute 2006 Scientific Symposium: Winning the War Against Cancer.** Grand Rapids, MI Sept 10-12.

Presented by Dr. Miranti:

Sridhar, S.C, Knudsen, B.S., and Miranti, C.K. 2004. Metastasis Suppressor CD82 Regulates Integrin-Mediated Signaling in Primary and Prostate Tumor Cells. **Gordon Conference: Signaling by Adhesion Receptors.** Bristol, RI, June 20-25.

Sridhar, S., Knudsen, B.S., and Miranti, C.K. 2004. Metastasis suppressor CD82 regulates integrin-mediated signaling in primary and prostate tumor cells. **Society for Basic Urologic Research 14th annual meeting: Signaling Mechanisms in Cell Proliferation and Death.** Savannah, GA, Dec. 9-12.

Edick, M.E., Knudsen, B.S. and Miranti, C.K. 2005. Integrin-mediated survival in primary prostate epithelial cells requires EGFR and c-Met. **Cold Spring Harbor 70th Symposium: Cancer.** Cold Spring Harbor, NY, June 1-6.

Edick, M.J., Lamb, L.E., Knudsen, B.S. and Miranti, C.K. 2005. Integrin-mediated survival in primary prostate epithelial cells requires EGFR and c-Met. **FASEB: Growth Factor Receptor Tyrosine Kinases in Mitogenesis, Morphogenesis, and Tumorigenesis**, Tucson, AZ, August 6-11.

Mathew J. Edick, Laura E. Lamb, Beatrice S. Knudsen and Cindy K. Miranti. 2006. Survival of Prostate Epithelial Cells on Laminin Requires Integrin-Mediated Activation of the EGFR/Erk and Src Signaling Pathways to Maintain Bcl-XL Expression. **Gordon Conference: Signaling by Adhesion Receptors.** Mount Holyoke, MA, June 25-30.

Mathew J. Edick, Laura E. Lamb, Beatrice S. Knudsen, and Cindy K. Miranti. 2006. Autophagy-Mediated Survival of Prostate Epithelial Cells on Laminin Requires Integrin-Mediated Activation of the EGFR/Erk and Src Signaling Pathways to Maintain Bcl-XL Expression. **AACR: Innovations in Prostate Cancer Research.** San Francisco, CA, December 6-9.

Mathew J. Edick, Laura E. Lamb, Beatrice S. Knudsen, and Cindy K. Miranti. 2006. Autophagy-Mediated Survival of Prostate Epithelial Cells on Laminin Requires Integrin-Mediated Activation of the EGFR/Erk and Src Signaling Pathways to Maintain Bcl-XL Expression. **46th Annual Meeting: American Society for Cell Biology.** San Diego, CA, Dec 9-13.

Miranti, C.K., Sridhar, S., Moshkovitz, S, Saari, K.M., Tesfay, L, and Edick, M.J. 2007. The Prostate Cancer Metastasis Gene, KAI1/CD82, Suppresses Tumor Cell Invasion and Metastasis through Regulation of the Receptor Tyrosine Kinase c-Met. **DOD PCRP IMPaCT Meeting**, Atlanta, GA, Sept 5-8.

4. We have generated the following reagents/models:

a. Stable cell lines of PC3 and DU145 cells expressing CD82 and/or c-Met
b. An orthotopic metastatic cancer model for PC3 cells in nude mice
c. Adenoviruses that express an shRNA specific to CD82 to inhibit its expression
d. Adenoviruses that express the androgen receptor
e. Stable PC3 and DU145 cell lines expressing AR
f. Differentiation model in PECs that will allow better understanding of AR function.
g. Stable PC3 cells lines that express chimeric mutants of CD82 and EC2 domain mutants.
h. a transgenic mouse over expressing human HGF has been used to generate an orthotopic metastastic prostate cancer model using DU145 cells.
i. mice containing floxed alleles of CD82 alone or CD82 and Pten together.
j. conditional loss of CD82 expression in all tissues of the mouse

5. We have applied for the following funding:

2004: Dr. Suganthi Sridhar applied for an NRSA fellowship from NIH.
It was not funded

2005: Two grants were applied for and funded:

108573 Research Scholar Grant (Miranti, PI) 7/1/05-6/30/09
American Cancer Society
Integrin and RTK Signaling and Crosstalk in Prostate Epithelial Cells
Role in Project: PI
The goal of this project is to determine the role of integrins and receptor tyrosine kinases in regulating signaling, cell survival, and proliferation of primary prostate epithelia cells.

Elsa Pardee Foundation Grant (Miranti, PI) 9/1/05-8/31/07
Elsa U. Pardee Foundation
Conditional Loss of the Metastasis Suppressor KAI1/CD82 in the Prostate
Role in Project: PI
The goal of this project is to generate conditional loss of expression of CD82 in the prostate of mice so that we can determine the role of CD82 in prostate tumor metastasis in vivo.

2006: NIH R01 application was submitted in June 2006 to extend our studies begun with the DOD New Investigator Award. It was not scored. It was revised and resubmitted July 2007.

Association for International Cancer Research application was submitted in October 2006 for a prostate-specific grant based on our CD82 studies. It was not funded, but will be resubmitted in October 2007.

2007: DOD Idea Award application was submitted in April 2007. It is currently under review.

6. Dr. Suganthi Sridhar, the postdoctoral fellow who is supported by this grant, applied for and accepted an Assistant Professor position in the Department of Biomedical Sciences at Grand Valley State University in 2006.

7. Dr. Miranti was asked to help organize the next FASEB: Tetraspanin Meeting in 2008.

8. Dr. Miranti has served as scientific reviewer for the DOD PCRP for three years.

CONCLUSIONS

Prior to our studies, the role of CD82 loss in regulating prostate tumor metastasis had not been determined. We have demonstrated that in metastatic tumor cells where elevated c-Met expression and activation by integrins is responsible for enhancing laminin-dependent migration and invasion, re-expression of CD82 suppresses both c-Met and Src signaling. This is not due to changes in integrin expression on the cell surface. Signaling through both Src and c-Met is required for cell migration and invasion in metastatic tumor cells. Conversely, suppression of CD82 expression in normal cells increases c-Met activation by laminin. We have also shown that CD82 acts to modulate c-Met signaling in response to its ligand HGF/SF. At low levels of ligand, CD82 suppresses, but when ligand is sufficiently high, it can activate c-Met even if CD82 is present. We have further determined that CD82 regulates c-Met activation through an indirect mechanism by altering the distribution of c-Met on the surface of the cell. This may be due to the ability of CD82 to interact with CD9 and β1 integrins, and/or CD82-mediated regulation of a tyrosine phosphatase. When tumor cells are injected orthotopically into the prostates of mice, CD82 suppresses both metastasis and growth, even through CD82 expression has no effect on cell proliferation in vitro, suggesting specific effects of the prostate environment on CD82 function. We are currently developing a transgenic mouse model and a germline conditional loss of CD82 to test our hypothesis that CD82 regulates c-Met *in vivo*. We have identified the PI-3K/Akt pathway as a critical component of laminin-specific survival in metastatic prostate cells and shown that expression of AR by-passes the need for PI-3K signaling when cells are adherent to laminin. We have developed an in vitro differentiation assay which allows us to generate primary cells, which already express CD82, to also express AR. These cells can be used to see how interactions between CD82 and AR are regulated.

So What: Our findings have broad implications for the control of metastatic cancer. CD82 loss has been reported in many types of cancers. Likewise, c-Met over expression, mutation, or activation has also been reported for a wide range of cancers and its aberrant activity correlates with the development of metastasis (14-16). We propose that loss of CD82 may be required for the development of metastasis, by removing a control point for c-Met signaling. We will be testing this idea in our CD82 deficient mice. In devising possible treatment strategies for metastatic cancer the replacement of a lost gene is much more difficult than pharmacologically suppressing the activity of an active gene. Therefore, our studies also demonstrate that targeting c-Met (or Src) would be a logical approach to therapeutic intervention. We are currently developing an orthotopic model for prostate cancer metastasis using a SCID transgenic HGF mouse. These studies will allow us to test whether CD82 expression *in vivo* is sufficient to suppress signaling through c-Met *in vivo* as our *in vitro* data indicates.

Our findings also suggest the possibility of using both CD82 expression and c-Met activation state as a potential biomarker pair for the prediction of metastatic disease. Loss of CD82 correlates highly with metastatic disease. If it can be demonstrated that c-Met activity is also up-regulated in tumors that eventually metastasize, then these might make good prognostic tools.

Our studies have also advanced the knowledge of how members of the tetraspanin family function. Interestingly, three other tetraspanins, CD151, CO-029, and kitenin, appear to behave opposite to CD82, in that their levels of expression and activity are elevated in tumors (61-63). Since tetraspanins are known to interact with each other, it is possible that loss of CD82 may act in part by enhancing the expression or activity of other tetraspanins. Our preliminary data indicate that expression of CD82 in tumor cells leads to the association of CD82 with CD9 and a resulting increase in CD9 association with β1 integrin. Loss of CD9 by siRNA prevents the ability of CD82 to suppress c-Met activation. This may account for our observation that CD82 regulates c-Met function through an indirect mechanism. This idea will be explored further. In addition we are also exploring the possibility that CD82 might regulate a c-Met tyrosine phosphatase.

Our findings that the PI-3K pathway contributes to integrin-mediated survival in metastatic cells is important because of the proposed role for laminin in tumor invasion and metastasis (1). These data would suggest that targeting the PI-3K pathway would be an effective therapeutic approach for metastatic cancer. However, we have further demonstrated that expression of the androgen receptor negates this dependence on PI-3K and alters the expression of integrins in both tumor and normal cells to favor laminin

integrins. These data suggest that since over 90% of metastatic cancers still express AR, targeting the PI-3K pathway alone would not be sufficient in a laminin-rich environment. It will be necessary to determine how AR expression suppresses the dependence on PI-3K to identify additional targets for therapeutic intervention. AR also up-regulated the level of bcl-XL in cells, suggesting it may be necessary to target Bcl2 family members in addition to PI-3K. In addition we have developed the first in vitro differentiation model for prostate epithelial cells that will allow us to further investigate the potential role of CD82 in regulating AR function in normal versus tumor cells.

PERSONNEL SUPPORTED BY GRANT

Dr. Cynthia Miranti	PI	15% effort
Dr. Suganthi Sridhar	Postdoctoral Fellow	80% effort until 7/31/2006
Gary Rajah	Undergraduate Student	100% effort starting 8/1/2006
Veronique Schulz	Senior Technician	40% effort starting 8/1/2006

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APPENDIX

Reprints

Sridhar, S.C. and Miranti, C.K. 2006. Tetraspanin KAI1/CD82 suppresses invasion by inhibiting integrin-dependent crosstalk with c-Met receptor and Src kinases. Oncogene, 25:2367–78.

Knudsen, B.S. and Miranti, C.K. 2006. The impact of cell adhesion changes on proliferation and survival during prostate cancer development and progression. J. Cell. Biochem., 99:345 - 361.

Edick, M.J., Tesfay, L., Lamb, L.E., Knudsen, B.S., and Miranti, C.K. 2006. Inhibition of Integrin-Mediated Crosstalk with EGFR/Erk or Src Signaling Pathways in Autophagic Prostate Epithelial Cells Induces Caspase-Independent Death. Mol. Biol. Cell, 18:2481-90.

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ORIGINAL ARTICLE

Tetraspanin KAI1/CD82 suppresses invasion by inhibiting integrin-dependent crosstalk with c-Met receptor and Src kinases

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KAI1/CD82, a tetraspanin protein, was first identified as a metastasis suppressor in prostate cancer. How loss of CD82 expression promotes cancer metastasis is unknown. Restoration of CD82 expression to physiological levels in the metastatic prostate cell line PC3 inhibits integrin-mediated cell migration and invasion, but does not affect integrin expression. Integrin-dependent activation of the receptor kinase c-Met is dramatically reduced in CD82-expressing cells, as is c-Met activation by its ligand HGF/SF. CD82 expression also reduced integrin-induced activation and phosphorylation of the cytoplasmic tyrosine kinase Src, and its downstream substrates p130Cas and FAK Y861. Inhibition of c-Met expression or Src kinase function reduced matrigel invasion of PC3 cells to the same extent as CD82 expression. These data indicate that CD82 functions to suppress integrin-induced invasion by regulating signaling to c-Met and Src kinases, and suggests that CD82 loss may promote metastasis by removing a negative regulator of c-Met and Src signaling.
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Keywords: tetraspanin; integrin; c-Met; HGF; metastasis

Introduction

KAI1/CD82 was originally identified as a suppressor of metastatic spread of tumor cells in a rat prostate model (Dong *et al*., 1995). Numerous subsequent studies demonstrated a direct correlation with loss of CD82 expression and poor prognosis in human prostate cancer (Dong *et al*., 1996; Sridhar and Miranti, 2005). Ubiquitous expression of CD82 prompted others to explore the possibility that loss of expression may not be limited to the prostate. Loss or low expression has been shown to correlate with poor prognosis in lung (Adachi *et al*., 1996), pancreatic (Guo *et al*., 1996), breast (Huang *et al*., 1998), bladder (Yu *et al*., 1997), colon (Lombardi *et al*., 1999), esophageal (Uchida *et al*., 1999; Miyazaki *et al*., 2000), cervical (Liu *et al*., 2001), ovarian (Liu *et al*., 2000), and endometrial (Wu *et al*., 2003) cancers. Downregulation of CD82 expression has also been observed in many metastatic tumor cell lines (White *et al*., 1998).

KAI1/CD82 belongs to the transmembrane 4 super family (TM4SF), also referred to as tetraspanins (Sridhar and Miranti, 2005). Tetraspanins have been implicated in the regulation of cell motility, morphology, fusion, signaling, fertilization, and differentiation (Maecker *et al*., 1997; Boucheix and Rubinstein, 2001; Hemler, 2001, 2003). In addition to CD82, loss of expression of tetraspanin molecules CD9 or CD63 also correlates with poor prognosis and increased metastasis (Higashiyama *et al*., 1995; Radford *et al*., 1995; Boucheix *et al*., 2001). Enhanced expression of tetraspanins CD81 and CD151, on the other hand, have been shown to correlate with poor prognosis and contribute significantly to increased cell motility and metastasis (Testa *et al*., 1999; Owens and Watt, 2001; Tokuhara *et al*., 2001). Tetraspanins lack intrinsic activity and their effects are mediated by protein interactions. Several different proteins have been shown to associate with CD82 (Yunta and Lazo, 2003), including $\alpha3\beta1$, $\alpha4\beta1$, $\alpha5\beta1$, and $\alpha6\beta1$ integrins (Berditchevski and Odintsova, 1999; Berditchevski, 2001; Yunta and Lazo, 2003); the serine/threonine kinase PKCα (Berditchevski and Odintsova, 1999; Berditchevski, 2001; Zhang *et al*., 2001); B-cell receptors CD4, CD8, and CD19 (Imai *et al*., 1995; Mannion *et al*., 1996; Hammond *et al*., 1998); other tetraspanins including CD81, CD63, and CD9 (Vogt *et al*., 2002); receptor tyrosine kinases EGFR and ErbB2 (Odintsova *et al*., 2000, 2003); an Ig super family molecule EW12 (Zhang *et al*., 2003b); and a newly identified tetraspanin molecule, kitenin, which is expressed in gastric cancers (Lee *et al*., 2004b).

Integrins are heterodimeric transmembrane proteins that link the extracellular matrix to intracellular cytoskeletal structures and signaling molecules (Miranti and Brugge, 2002). Changes in integrin levels have been reported to occur during tumorigenesis and metastasis of many tumors (Clezardin, 1998). In prostate cancer, the reported changes in integrins include increased expression levels of $\alpha3\beta1$ and $\alpha6\beta1$ (Schmelz *et al*., 2002), loss of $\beta4$ (Davis *et al*., 2001), and a decrease in $\alpha5\beta1$c (Perlino *et al*., 2000) and $\alpha2\beta1$ integrins (Dong *et al*., 1997). Integrins are implicated in regulating

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cellular processes such as adhesion, signaling, growth and differentiation, motility, survival, and gene expression (Miranti and Brugge, 2002). Both integrins and receptor tyrosine kinases are involved in cell migration and metastasis. Furthermore, integrins have been shown to activate several receptor tyrosine kinases in a ligand-independent manner, including EGFR, c-Met, PDGFR, and Ron (Sundberg and Rubin, 1996; Wang *et al*., 1996, 2001; Moro *et al*., 1998, 2002; Danilkovitch-Miagkova *et al*., 2000; Bill *et al*., 2004). Both integrins and receptor tyrosine kinases can interact with CD82 (Hemler, 1998; Odintsova *et al*., 2000). Therefore, one mechanism by which CD82 may control tumor metastasis is by regulating the function of integrins and their ability to cooperate with receptor tyrosine kinases.

CD82 has been demonstrated to regulate the activity of the receptor tyrosine kinase, EGFR. EGF-mediated signaling and wound closure were shown to be attenuated after ectopic expression of CD82 in a breast tumor cell line (Odintsova *et al*., 2000). Accelerated endocytosis of the EGFR–EGF complex was suggested to be the cause for signal attenuation. Unlike breast cancer, a role for EGFR family members in prostate metastatic disease is less frequently observed. The tyrosine kinase receptor for hepatocyte growth factor/scatter factor (HGF/SF), c-Met, on the other hand, has been implicated in promoting prostate cancer metastasis. Overexpression of c-Met has been detected in prostate cancer and is associated with conversion to androgen independence and invasion (Fixman *et al*., 1995; Humphrey *et al*., 1995; Maggiora *et al*., 1997; Nakashiro *et al*., 2003; Knudsen and Edlund, 2004). Src signaling downstream of c-Met may regulate tumor metastasis through regulation of migration and invasion (Ponzetto *et al*., 1994; Rahimi *et al*., 1998; Mora *et al*., 2002; Kim *et al*., 2003). Src substrates involved in migration and invasion include focal adhesion kinase (FAK) and p130Cas (Cary *et al*., 1998). Inhibition of p130Cas–Crk coupling has been suggested to be required for CD82-mediated inhibition of cell migration in CD82-expressing DU145 cells (Zhang *et al*., 2003a). These findings suggest that integrin and receptor tyrosine kinase signalling may be a target for CD82.

To better understand how CD82 is involved in regulating integrin- and receptor tyrosine kinase-mediated signaling, we have re-expressed CD82 in a highly invasive prostate cancer cell line PC3. We have examined CD82 regulation of integrin-mediated activation of c-Met and Src family kinases and the downstream signaling molecules involved in migration, invasion, and metastasis. These studies provide a better understanding of how loss of CD82 promotes prostate cancer metastasis.

Results

CD82 expression in PC3 cells

To characterize the role of CD82 in suppressing tumor progression and metastasis, PC3 cells were stably transfected with a CD82 cDNA. Vector-transfected stable clones were generated as controls. Figure 1a shows CD82 expression in the lysates from parental PC3 cells, seven CD82 clones (CL# 3, 6, 11, 12, 13, 14, and 29), and one vector clone (5V) after immunoblotting with anti-CD82 antibodies. The level of expression of CD82 in these tumor cell clones was comparable to the level of expression in primary prostate epithelial cells (PEC). Surface expression of CD82 was confirmed by FACS analysis (Figure 1b) and immunofluorescent staining (Figure 1c).

CD82 expression reduces migration and matrigel invasion

CD82 is known to associate with $\alpha3\beta1$ and $\alpha6\beta1$ integrins (Hemler, 1998; Berditchevski and Odintsova, 1999; Ono *et al*., 2000), integrins specifically involved in adhesion to laminin. No differences in adhesion to laminin (or other matrices) between the vector- and CD82-transfected clones was observed over a 100-fold range in laminin concentrations (Figure 2a). No cells adhered below 0.156 μg/ml laminin and a similar small number of cells (<100) adhered to 0.31 μg/ml independent of CD82 expression. Similar results were obtained when the number of cells adhering over time was measured (not shown). There were also no changes in surface expression of laminin-binding integrins $\alpha3$ (not shown), $\alpha6$, $\beta4$, or $\beta1$ on any of eight clones tested, as measured by FACS or biotin labeling (Figure 2b, c). PC3 cells are elongated and spindle-shaped. Expression of CD82 resulted in rounder, more cuboidal-shaped cells resembling PECs (data not shown). PC3 cells expressing

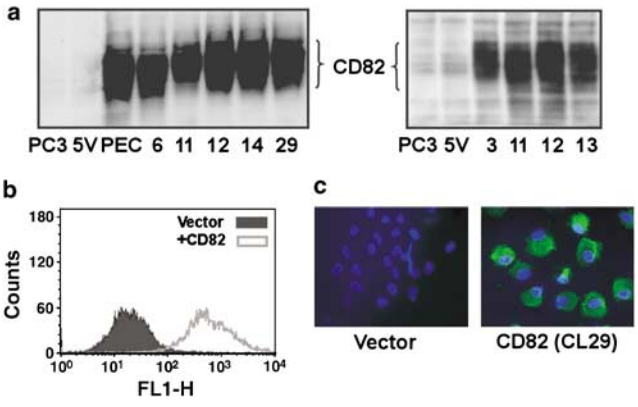

Figure 1 CD82 expression in PC3 cells. (**a**) Immunoblots of lysates from primary prostate epithelial cells (PEC), parental PC3 cells, vector-transfected cells (5V), and CD82-expressing PC3 clones (CL# 3, 6, 11–14, and 29) picked after stable transfection with CD82 cDNA and selection in 2 μg/ml puromycin. (**b**) Surface expression of CD82 in vector and one of the representative CD82-expressing clones was analysed by FACS following staining with anti-CD82 antibodies and FITC-conjugated secondary antibody. (**c**) Immunofluorescent staining of vector- or CD82-transfected stable clone #29 (CL29) adherent to collagen with anti-CD82 antibodies followed by staining with FITC-conjugated secondary antibody (green) and nuclear counterstaining with Hoechst 33258 (blue). Vector-transfected cells have no detectable CD82 expression (only nuclei are visible), while CD82-transfected cells (green) express CD82 in the membrane and cytoplasm surrounding the nuclei.

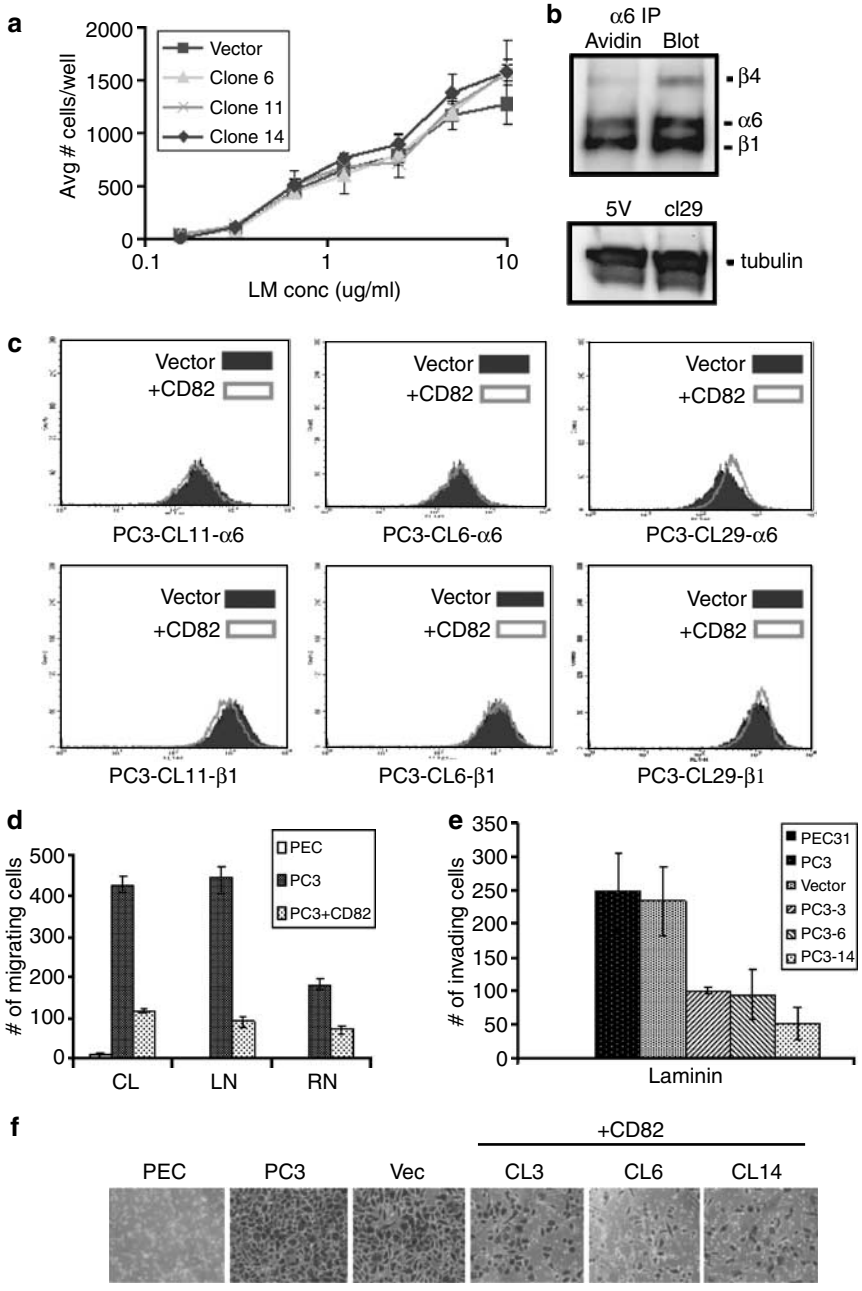

Figure 2 CD82 alters PC3 cell migration and invasion, but not integrin expression. (**a**) PC3 cells expressing CD82 (clone 6, 11, or 14) or vector were plated in triplicate on the indicated concentrations of laminin for 45 min. Nonadherent cells were washed away and stained. The number of adherent cells was counted in two random fields per well, averaged, and the standard deviation calculated. There were no significant differences between the cell lines. (**b**) PC3 cells expressing CD82 (cl29) or vector growing in culture was labeled with biotin at 4°C. Integrin α6 was immunoprecipitated and the levels of biotin labeling were monitored by blotting with anti-avidin. Total levels of protein in the extracts were equal as indicated by immunoblotting for tubulin (Tub Blot). Both β1 and β4 integrins were detected in the α6 immunoprecipitates. (**c**) Cell surface expression of α6 and β1 integrins in vector and three CD82-expressing PC3 clones (CL# 6, 11, 29) by FACS. (**d**) PC3 cells stably transfected with vector (PC3) or CD82 cDNA along with primary prostate epithelial cells (PEC) were analysed for their ability to migrate towards collagen, laminin, or fibronectin in 8 μm Boyden chambers in the absence of growth factors or serum. Cells passing through the pores were stained with crystal violet and counted. (**e**, **f**) CD82-expressing PC3 clones (CL# 3, 6, 14), vector-transfected cells (Vec), parental cells (PC3), or primary cells (PEC) were tested for their ability to invade matrigel through 8 μm Boyden chambers to laminin-coated membranes. Cells that invaded and passed through the membrane were stained, photographed, and counted.

CD82 also displayed reduced migration towards laminin and collagen (Figure 2d).

The ability of CD82-expressing PC3 cells to invade matrigel and extrude through 8 μm-pored membranes as an *in vitro* measurement of invasiveness was also tested. Parental or PC3 cells stably transfected with vector, but not primary cells (PECs), were able to invade the matrigel in the absence of serum or growth factors

(Figure 2e, f). All clones expressing CD82, however, demonstrated a dramatic reduction in their invasive ability. These experiments clearly indicate that CD82 expression not only reduces migration but also lowers *in vitro* invasiveness in this highly invasive cell line. FACS analysis indicated there were also no changes in surface expression of other integrins including α2, α3, or α5 (not shown). Therefore, the effects of CD82 expression on cell migration and invasion is likely due to changes in signaling downstream of integrin-mediated adhesion.

CD82 regulates integrin- and ligand-induced activation of c-Met

One mechanism by which tetraspanins may control metastasis is by regulating the function of tyrosine kinases involved in cell migration and metastasis. To investigate this possibility, c-Met activation upon integrin and ligand stimulation in vector- or CD82-expressing cells was monitored. Adhesion of PC3 cells to collagen, laminin, or fibronectin, in the absence of exogenous growth factors or serum, induced tyrosine phosphorylation of c-Met. CD82 expression suppressed integrin-induced c-Met activation in all CD82-expressing PC3 clones (Figure 3a).

Vector- and CD82-transfected clones were treated with the c-Met ligand HGF/SF at different concentrations and the effect on ligand-induced activation of c-Met was monitored. Higher concentrations of HGF/SF were required in the CD82-expressing cells to achieve the same level of c-Met tyrosine phosphorylation observed at lower concentrations with vector control cells (Figure 3b). These results indicate that CD82 can regulate both integrin- and ligand-mediated activation of c-Met in PC3 cells.

CD82 does not appear to be regulating the turnover of c-Met protein. Total protein levels of c-Met, as determined by immunoblotting, were relatively unchanged. Neither were there any differences in the levels of c-Met protein over time following integrin- or ligand-induced c-Met activation between vector and CD82-expressing cells (see Supplementary data, Figure S2B). Furthermore, no significant changes in cell surface expression of c-Met by FACS analysis (Figure S2A) were detected. Thus, CD82 acts to suppress the extent of c-Met activation, rather than affecting the overall levels of c-Met. To determine if c-Met and CD82 can associate with each other, their ability to colocalize within cells was assessed by immunostaining and confocal microscopy. We were unable to detect colocalization of c-Met and CD82 at either the surface or within the cell (Figure 3c). We were also unable to detect an association by co-immunoprecipitation (not shown). These data suggest that CD82 affects c-Met activation through an indirect mechanism.

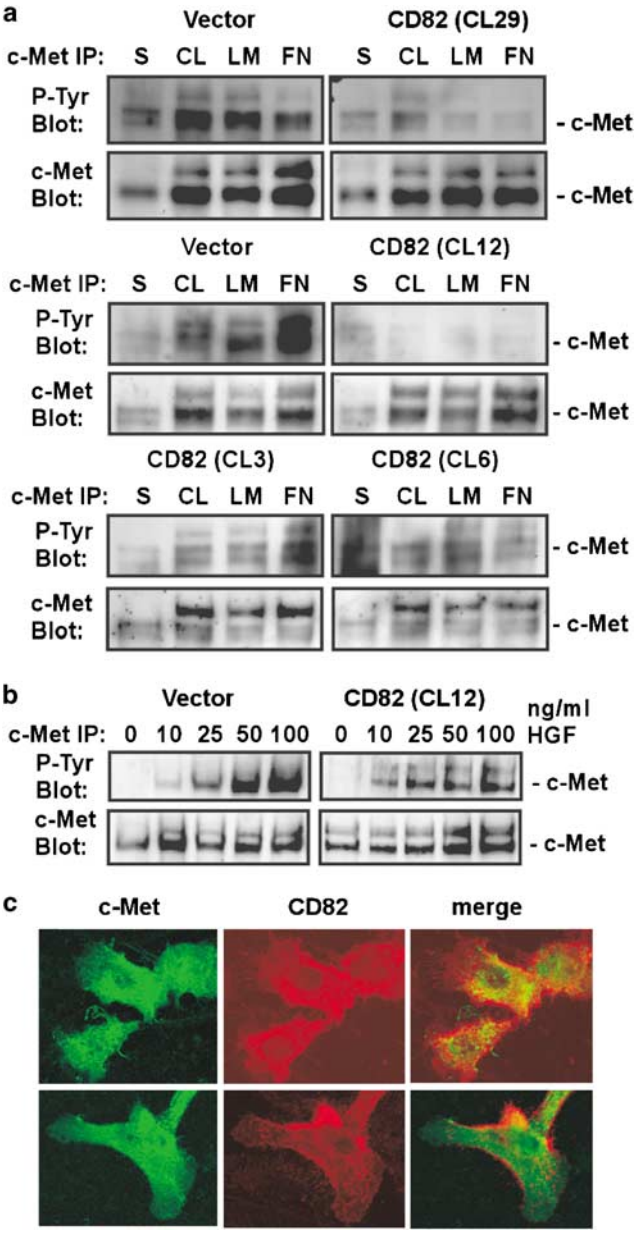

Figure 3 CD82 regulates integrin- and ligand-induced activation of c-Met. (**a**) PC3 clones (CL# 3, 6, 12, 29) stably transfected with CD82 or vector were placed in suspension (S) or plated on collagen (CL), laminin (LM), or fibronectin (FN) in the absence of exogenous growth factors or serum for 1 h. (**b**) PC3 cells stably transfected with CD82 (CL12) or vector were serum starved and then stimulated with 0, 10, 25, 50, or 100 ng/ml HGF/SF for 5 min. (**a**, **b**) The levels of c-Met phosphorylation were measured by immunoblotting of immunoprecipitates with anti-phosphotyrosine antibodies (P-Tyr Blot) followed by probing stripped blots with anti-Met antibodies (c-Met Blot). (**c**) Immunostaining of CD82-expressing cells with anti-c-Met (green) and anti-CD82 (red) antibodies. No significant colocalization (merge) was detected.

CD82 regulates phosphorylation of Src and its substrates FAK and p130Cas

Integrins and c-Met regulate cell migration and invasion by regulating downstream signaling pathways such as Src, PI-3K/Akt, and Ras/Erk. Integrin-mediated adhesion of PC3 cells does not lead to Erk activation and the PI-3K pathway is constitutively activated (see Supplementary data, Figure S3A). Furthermore, re-expression of CD82 did not alter signaling to Erk or to Akt (Figure S3B). Both integrins and c-Met have been shown to be effective in activating Src (Rahimi *et al*.,

1998; Nakaigawa *et al*., 2000; Sieg *et al*., 2000; Timpson *et al*., 2001; Hauck *et al*., 2002; Hsia *et al*., 2003; Frame, 2004; Westhoff *et al*., 2004; Brunton *et al*., 2005). To determine if CD82 is involved in signaling to Src, we investigated Src activation following integrin engagement in CD82-expressing cells. Src phosphorylation at the activation loop Y418 was monitored by immunoblotting with a phospho-specific antibody. Src activation was reduced upon integrin engagement in CD82-expressing cells (Figure 4a). To further support these findings, Src substrates implicated in cell migration and invasion, FAK and p130Cas, were monitored. The levels of p130Cas protein were frequently observed to decrease when cells were placed in suspension independent of CD82 expression. Nonetheless, the presence of CD82 inhibited tyrosine phosphorylation of the Src substrate p130Cas (Figure 4b) when cells were replated on matrix. Expression of CD82 in PC3 cells also reduced integrin-induced phosphorylation at the Src-specific phosphorylation site Y861 in FAK, but not Y397, the FAK autophosphorylation site (Figure 4c). Primary PEC express significant quantities of CD82 (Figure 1a). Correspondingly, there is reduced integrin-dependent activation of Src and p130Cas, in PECs compared to PC3 tumor cells (Figure 4a, b). Thus, CD82 may limit Src signaling in normal cells and its loss in tumor cells may enhance signaling through Src.

Inhibition of Src or c-Met blocks invasion

CD82 expression in PC3 cells reduces matrigel invasion and also reduces activation of c-Met and Src. Therefore, we tested whether c-Met and Src are also required for invasion. Parental or vector PC3 cells were pretreated with the Src kinase-specific inhibitor, SU6656 (Blake *et al*., 2000), and their ability to invade matrigel was monitored. Inhibition of Src activity blocked invasion to the same extent as CD82 expression (Figure 5a, b). Furthermore, treatment of CD82-expressing cells with SU6656 did not further inhibit invasion, indicating that the level of Src suppression achieved by CD82 expression was equivalent to that obtained by directly inhibiting Src kinases.

The lack of available specific pharmacological inhibitors of c-Met prompted the use of siRNA strategies to inhibit c-Met activity in tumor cells. Infection of PC3, DU145, or primary cells with c-Met shRNA containing adenoviruses at an m.o.i. of 100 resulted in nearly complete suppression of c-Met expression within 2–3 days (Figure 5c). PC3 cells expressing c-Met si-hMet-Ad[221] displayed a reduced level of matrigel invasion (Figure 5d, e); the levels were similar to that observed with the Src inhibitor. Similar results were obtained using another c-Met shRNA, si-mMet-Ad[178] (not shown). Thus, both Src and c-Met contribute to the invasive phenotype of PC3 cells, both of which are suppressed by CD82.

CD82 separately suppresses c-Met and Src signaling

Inhibition of Src or c-Met signaling by CD82, to suppress invasion, could be due to two separate signaling events, or Src and c-Met could be influencing the others activity. Integrin-mediated activation of EGFR and Ron (a close relative to c-Met) has been shown to be dependent on Src activation (Danilkovitch-Miagkova *et al*., 2000; Moro *et al*., 2002). Whether

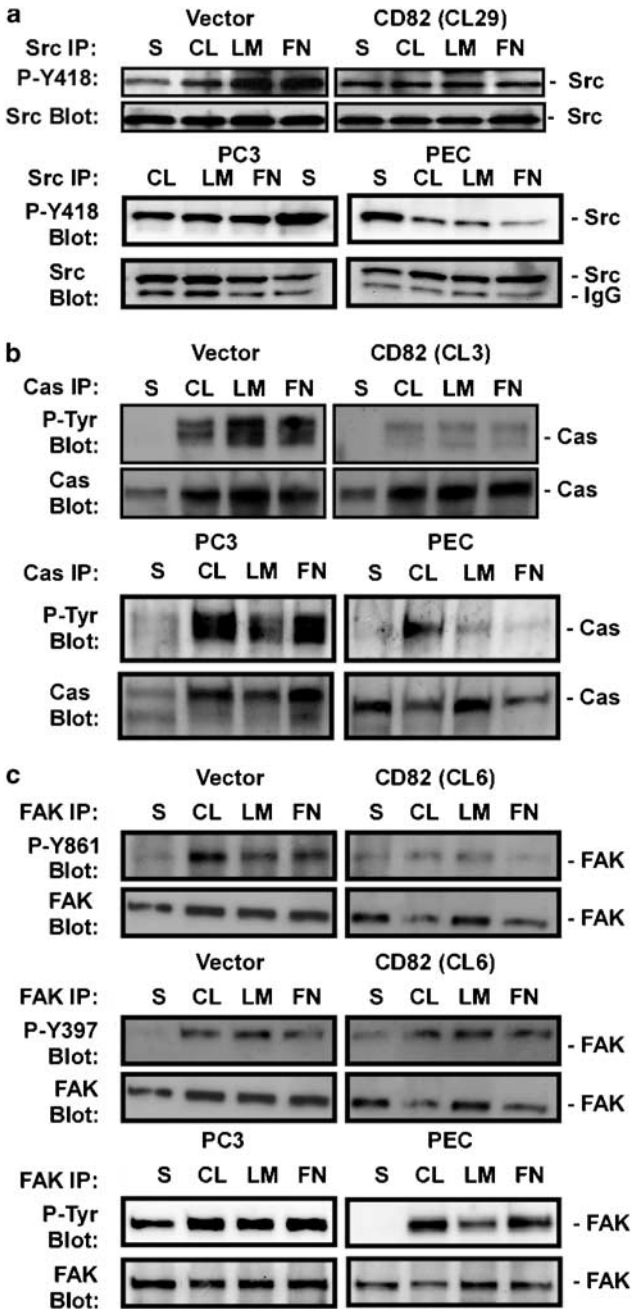

Figure 4 CD82 regulates phosphorylation of Src and its substrates FAK and p130Cas. Vector or CD82 stably transfected PC3 clones (CL# 3, 6 29), parental PC3 cells (PC3), or primary cells (PEC) were placed in suspension (S) or plated on collagen (CL), laminin (LM), or fibronectin (FN). After 1 h, the levels of (**a**) Src Y418, (**b**) p130Cas, (**c**) FAK Y861 or FAK Y397 phosphorylation was measured by immunoblotting of immunoprecipitates with respective phospho-specific or anti-phosphotyrosine antibodies (P-Y418, P-Tyr, P-Y861, or P-Y397 Blots). Total levels of protein in the immunoprecipitates were measured by immunoblotting of stripped blots with respective antibodies (Src, Cas, and FAK Blots).

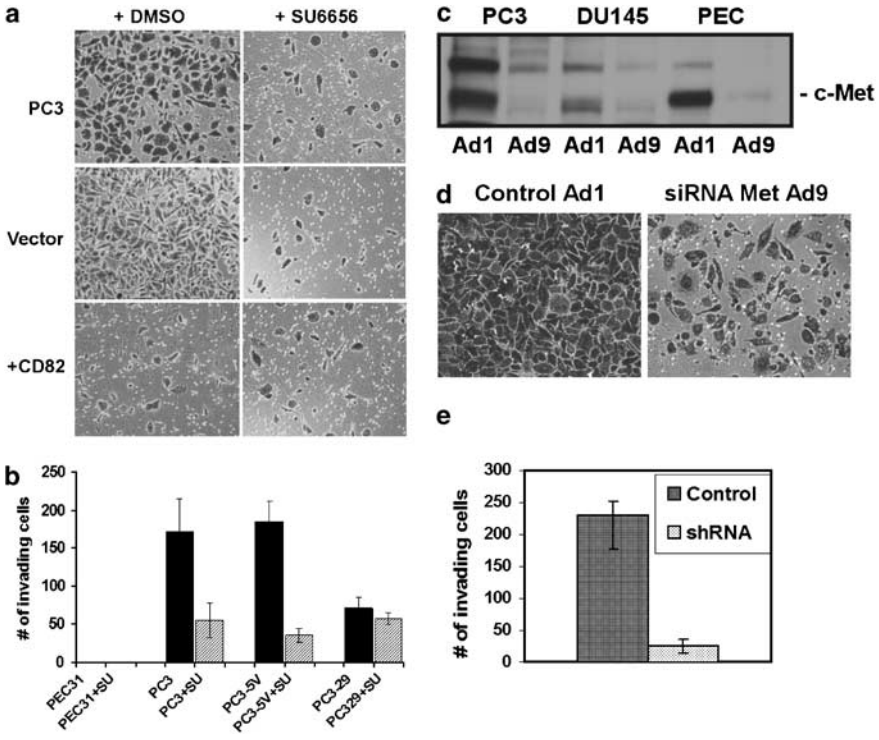

Figure 5 Inhibition of Src or c-Met blocks invasion. (**a**) Parental (PC3), vector, or CD82 stably transfected clones were untreated (DMSO) or treated with 2 μM of the Src inhibitor SU6656 and monitored for invasion through a matrigel. Cells that invade through the matrigel and pass through the 8 μm pores were stained with crystal violet, photographed, and counted. (**b**) Quantification of Src-mediated invasion. (**c**) PC3, DU145, or primary prostate epithelial cells (PEC) were infected with control (Ad1) or c-Met shRNA si-hMet-Ad^{221} Adenovirus (Ad9). After 3 days, the levels of c-Met expression were monitored by immunoblotting. (**d**) Control or c-Met shRNA-infected PC3 cells were tested for their ability to invade matrigel as described in (**a**). (**e**) Quantification of c-Met-mediated cell invasion.

c-Met activation might be involved in regulating integrin-mediated activation of Src is unknown. To test these models, Src activation was blocked with 2 μM SU6656 (Blake *et al*., 2000) and the effect on c-Met activation following integrin engagement was monitored. Blocking Src did not change integrin-mediated c-Met activation, but did block integrin-induced p130Cas phosphorylation (a Src substrate) (Figure 6a, b). Inhibition of c-Met by si-hMet-Ad^{221} or si-mMet-Ad^{178} expression did not block integrin-mediated activation of Src, as measured by phosphorylation at Y418 (Figure 6c). Thus, integrin-mediated activation of Src and c-Met in PC3 cells are independent events. However, both are required for matrigel invasion (Figure 5).

Overexpression of c-Met induces invasion

To determine if CD82 expression in another prostate tumor cell line can also suppress c-Met activation, we selected stable CD82 transfectants of DU145 cells (Figure 7b). Surprisingly, c-Met was not activated in DU145 cells by plating on matrix (Figure 7a). Immunoblotting indicated that the levels of c-Met expression in DU145 cells are greatly reduced compared to normal PECs or PC3 cells (Figure 5c). Correspondingly, DU145 cells, unlike PC3 cells, failed to invade matrigel in the absence of serum or growth factors (Figure 7c, d). Treatment with HGF was sufficient to promote DU145 invasion, but did not increase PC3 cell matrigel invasion. To better understand CD82 regulation of c-Met, we overexpressed c-Met in DU145 cells and in CD82-expressing DU145 cells (Figure 7b). Expression of c-Met in DU145 cells was sufficient to permit matrigel invasion in the absence of HGF (Figure 7a). Expression of CD82 suppressed HGF-induced invasion at low concentrations of HGF. High concentrations of HGF overcame the inhibitory effects of CD82 expression. Thus, CD82 affects the relative sensitivity of signaling through c-Met.

Discussion

The results from this study indicate that re-expression of CD82 in a highly invasive metastatic prostate cancer cell line, PC3, suppresses (1) cell migration on laminin and collagen, (2) both integrin- and ligand-induced activation of c-Met, (3) integrin-mediated activation of Src and two of its downstream substrates p130Cas and FAK Y861, and (4) both integrin- and HGF-mediated invasion of laminin-rich matrigel. Furthermore, inhibition of Src or c-Met function reduced integrin-mediated invasion to the same extent as CD82 expression. Overexpression of c-Met was sufficient to induce

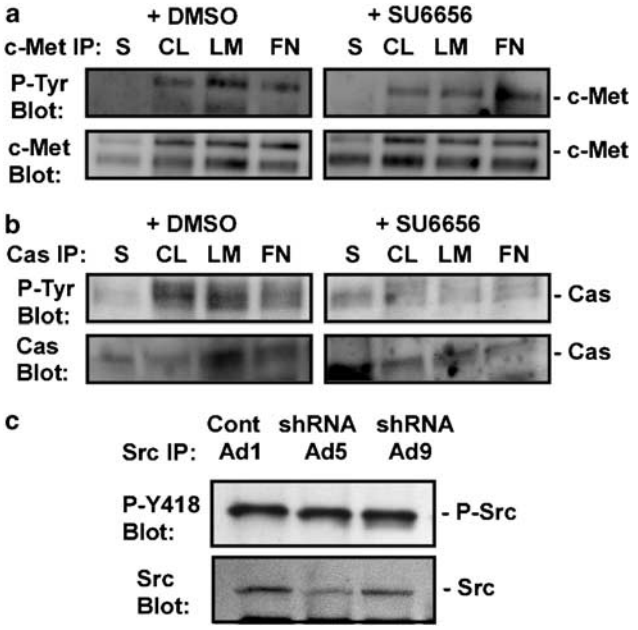

Figure 6 CD82 suppresses c-Met and Src signaling independently. (**a**) Parental PC3 cells were untreated (DMSO) or treated with the 2 μM of the Src inhibitor SU6656. Cells were then placed in suspension (S) or plated on collagen (CL), laminin (LM), or fibronectin (FN). The levels of c-Met activation were measured by immunoblotting of c-Met immunoprecipitates with anti-phosphotyrosine antibodies (P-Tyr Blot). (**b**) The levels of p130Cas tyrosine phosphorylation were measured by immunoblotting of immunoprecipitates (P-Tyr Blot). Total levels of c-Met and Cas in the immunoprecipitates were monitored by immunoblotting of stripped blots with respective antibodies (c-Met, Cas Blots). (**c**) PC3 cells were infected at an m.o.i. of 100 with empty virus (Ad1) or two c-Met shRNAs, si-mMet-Ad^{175} (Ad5) or si-hMet-Ad^{221} (Ad9). The cells were plated on laminin (LM) and the levels of Src activation (P-Y418 Blot) in immunoprecipitates were analysed by immunoblotting with anti-Src Y418-phospho-specific antibodies, respectively. Total levels of Src in the immunoprecipitates were monitored by immunoblotting with anti-Src antibodies (Src Blot).

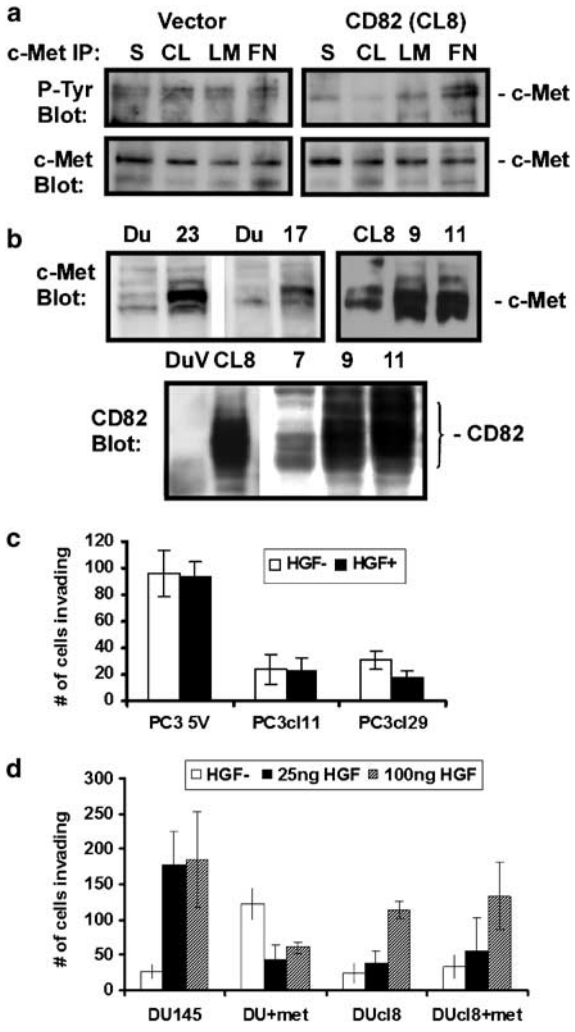

Figure 7 CD82 regulates HGF-mediated invasion. (**a**) DU145 cells stably transfected with CD82 (CL# 8) or vector were placed in suspension (S) or plated on collagen (CL), laminin (LM), or fibronectin (FN). After 1 h, the levels of c-Met tyrosine phosphorylation were measured by immunoblotting of immunoprecipitates with anti-phosphotyrosine antibodies (P-Tyr Blot). Total levels of protein in the immunoprecipitates were measured by immunoblotting of stripped blots with anti-c-Met antibodies (c-Met Blot). (**b**) Parental DU145 cells (Du) or CD82-expressing cells (CL8) were stably transfected with human c-Met cDNA. The levels of c-Met (c-Met Blot) in DU145 cells (Du), DU145 c-Met clones (23, 17), and CD82 CL8 clones (9, 11) and the levels of CD82 (CD82 Blot) in CL8, CL8 clones (7, 9, 11), and vector-transfected cells (DuV) were monitored by immunoblotting. (**c**) PC3 clones (cl11, cl29) stably transfected with CD82 or vector (5V) were tested for their ability to invade matrigel through 8 μm Boyden chambers to a laminin-coated membrane in the absence (HGF−) or presence of 25 ng/ml HGF. Cells that invaded and passed through the membrane were stained and counted. (**d**) Parental DU145 cells (DU145) or DU145 cells over expressing c-Met (DU + met), CD82 (DUcl8), or both c-Met and CD82 (DUcl8 + met) were allowed to invade matrigel in the absence (HGF−) or presence of 25 or 100 ng/ml HGF. Cells that invaded were stained and counted.

HGF-independent invasion and CD82 expression suppressed c-Met-mediated invasion. Based on these results, we propose a model whereby CD82 functions to suppress integrin-induced invasion by regulating c-Met and Src kinases (Figure 8). These data suggest that loss of CD82 expression in prostate tumors promotes metastasis by permitting more efficient activation of Src and c-Met by both integrins and ligand.

CD82, like other tetraspanins, has been reported to associate with several integrins, namely $\alpha3\beta1$, $\alpha4\beta1$, $\alpha5\beta1$, and $\alpha6\beta1$ (Berditchevski and Odintsova, 1999; Berditchevski, 2001; Yunta and Lazo, 2003). Previous studies have shown that the role of tetraspanins is not to regulate integrin expression, but to modulate integrin function and as such have been termed 'molecular facilitators' (Maecker *et al*., 1997; Hemler, 2001). However, a few papers have suggested that CD82 can regulate cell adhesion to fibronectin (Takaoka *et al*., 1998; Liu *et al*., 2003) or laminin (He *et al*., 2005). In one report, CD82 expression was found to downregulate the expression of $\alpha6$ integrin in DU145 cells (He *et al*., 2005). We monitored the cell surface expression of several different integrins in parental, vector, and eight different clones of CD82-expressing PC3 cells. We used two different methods, FACS and surface biotinylation, two different $\alpha6$ antibodies, and two different conditions, serum starved or adherent to laminin. We found

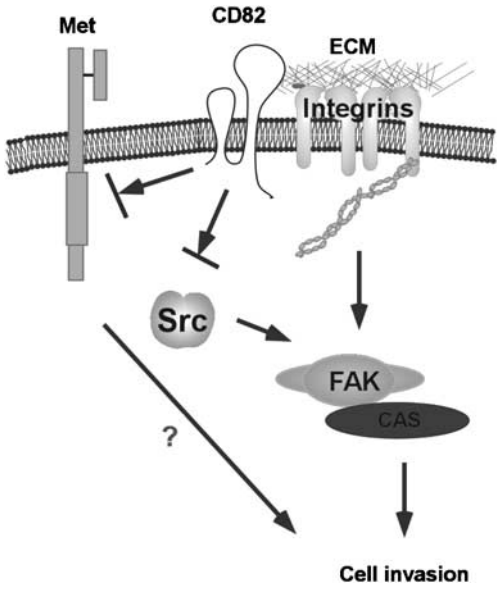

Figure 8 Model for CD82 regulation of invasion. CD82 functions to limit both integrin and ligand-induced activation of the receptor tyrosine kinase c-Met. Therefore, it is predicted that loss of CD82 during late-stage tumorigenesis permits increased activation of c-Met through both integrin and ligand. CD82 also independently decreases integrin-induced Src activation. Concurrently, increased signaling through Src leads to increased activation of downstream substrates, p130Cas and FAK, which regulate cell migration and invasion. The mechanism by which c-Met enhances invasion is not known.

no differences in integrin expression, not even with α6. We also did not observe any differences in the ability of the cells to adhere to different matrices including laminin. This discrepancy could be due to differences in cell lines. In our hands, DU145 cells adhere poorly to laminin and the levels of α6 integrin are much lower than in PC3 cells (see Supplementary data, Figure S1). DU145 cells also did not invade laminin-containing matrigel in the absence of serum or growth factors, but PC3 cells did. Another possibility is that the level of CD82 expression could vary between these studies. We selected clones that expressed similar or lower levels of CD82 than normal primary PEC. Overexpression of CD82 can lead to apoptosis (Schoenfeld *et al*., 2004). Thus our data indicate that in highly invasive cells that adhere, migrate, and invade well on laminin, CD82 expression does not change integrin expression, but rather acts downstream of integrin engagement.

c-Met, the receptor for HGF/SF, is known to regulate migration and invasion and has been implicated in promoting metastasis in many different kinds of cancers (Pisters *et al*., 1995; Birchmeier *et al*., 1997, 2003; Wang *et al*., 2001), including prostate cancer (Humphrey *et al*., 1995; Nishimura *et al*., 1998; Knudsen *et al*., 2002; van Leenders *et al*., 2002; Nakashiro *et al*., 2003; Knudsen and Edlund, 2004). Previous studies in a mammary tumor cell line demonstrated that CD82 expression attenuated EGF-mediated EGFR signaling and reduced migration (Odintsova *et al*., 2000). In PC3 or DU145 cells, we did not observe significant changes in integrin-induced activation of EGFR or EGFR levels due to CD82 expression (not shown). We have not measured the response of EGFR to EGF. We did observe a decrease in both integrin- and ligand-induced c-Met activation when CD82 is expressed, but neither resulted in a change in overall c-Met levels. These data suggest that there may be a fundamental difference between how EGFR and c-Met are regulated or that these cell types behave differently. In prostate cancer, c-Met, rather than EGFR or ErbB, has been the tyrosine kinase receptor implicated in metastasis (Humphrey *et al*., 1995; Pisters *et al*., 1995; Nishimura *et al*., 1998; Knudsen *et al*., 2002; van Leenders *et al*., 2002; Nakashiro *et al*., 2003; Knudsen and Edlund, 2004). The role of EGFR/Erb2 in prostate cancer, unlike breast cancer, remains controversial (Maygarden *et al*., 1992; De Miguel *et al*., 1999; Skacel *et al*., 2001; Di Lorenzo *et al*., 2002). Thus, CD82 may regulate different receptors in different cell or tumor types.

Previous studies have suggested that c-Met and laminin integrins may interact to regulate invasion (Trusolino *et al*., 2001; Chung *et al*., 2004). Our studies found that PC3 cells express higher levels of c-Met relative to DU145 cells and integrins were incapable of activating c-Met in DU145 cells. Consequently, in our assays, DU145 cells failed to invade through matrigel in the absence of serum or growth factors. However, we and others have demonstrated that DU145 cells will invade matrigel if HGF/SF is present (Nishimura *et al*., 1998, 1999; Davies *et al*., 2004). CD82 expression in DU145 cells was able to suppress HGF-mediated invasion. Low concentrations of HGF were not sufficient to overcome the effects of CD82. However, high concentrations of HGF reversed CD82-mediated suppression of invasion. Furthermore, overexpression of c-Met was sufficient to promote ligand-independent, integrin-mediated invasion of matrigel. These data strongly support the role of CD82 in regulating c-Met activation.

Signaling through Src has been proposed to be involved in regulating cell migration and invasion by integrins (Sieg *et al*., 2000; Hauck *et al*., 2002; Hsia *et al*., 2003; Frame, 2004; Westhoff *et al*., 2004; Brunton *et al*., 2005). Two downstream targets of Src, FAK and p130Cas, have been proposed to be required for integrin-mediated migration (Vuori *et al*., 1996; Klemke *et al*., 1998; Hauck *et al*., 2002). Our studies indicate that expression of CD82 in PC3 cells downregulates this pathway. A similar study in DU145 cells indicated that CD82 expression also modified this pathway, but through a different mechanism (Zhang *et al*., 2003a). While we observe a direct affect on Src signaling and its ability to efficiently phosphorylate down stream substrates, the study of Zhang *et al*. demonstrated no involvement of Src kinase activity, but rather a loss in p130Cas expression. These differences could be due to differences in cell lines, the integrins expressed, and the mechanisms they use to regulate cell movement. This may also reflect a difference in signaling pathways involved in regulating haptotactic migration on a two-dimensional surface versus the capacity to migrate and

invade in a three-dimensional network. Our studies have clearly demonstrated that Src is required for invasion and that CD82 suppresses both Src and invasion. Interestingly, expression of CD82 was not sufficient to completely block invasion (not to the level seen in normal cells), and neither was suppression of Src. In fact, inhibition of Src in the presence of CD82 did not further suppress invasion. These data indicate that other signaling events not involved in this pathway are also involved in regulating invasion. PC3 cells harbor a pTEN mutation which results in increased signaling through the PI-3K and Akt pathway. This pathway has also been linked to increased invasion (Tamura *et al*., 1999; Brader and Eccles, 2004). However, expression of CD82 failed to inhibit this signaling pathway, suggesting the possibility that this may be a non-CD82-regulated invasion pathway.

Our data support recent evidence for the existence of crosstalk between integrins and c-Met (Wang *et al*., 1996, 2001; Trusolino *et al*., 2001). Our data further suggest that CD82 may be regulating this crosstalk. The mechanism by which integrins activate receptor tyrosine kinases has not been completely resolved. One model proposes that Src activation by integrins leads to phosphorylation of the receptor cytoplasmic domain (Danilkovitch-Miagkova *et al*., 2000; Moro *et al*., 2002); however, this is not always the case (Bill *et al*., 2004). In PC3 cells, integrin-mediated activation of c-Met was not dependent on Src activity. Prior studies indicate that ligand-independent activation of receptor kinases is required for signaling downstream of integrins (Moro *et al*., 1998; Bill *et al*., 2004). However, we found that inhibition of c-Met had no effect on integrin-induced Src activation *per se*. We cannot rule out the possibility that c-Met could regulate Src activity indirectly by affecting its localization or ability to target downstream substrates.

Previous studies have suggested that CD82 may act positively to activate signaling to Src and FAK (Lagaudriere-Gesbert *et al*., 1998; Berditchevski and Odintsova, 1999; Jee *et al*., 2003). In T cells, anti-CD82 antibodies were shown to activate p56lck, a member of Src kinase family; however, this was only observed in the context of costimulation of the T-cell receptor and was not observed upon cell adhesion to anti-CD82 antibodies (Lagaudriere-Gesbert *et al*., 1998). In CD82-transfected DU145 cells, anti-CD82 antibody enhanced Src kinase activity in cell–cell aggregates in suspension independently of integrins (Jee *et al*., 2003). However, these two examples do not address the same biological question; what is the role of CD82 in regulating integrin functions. On the other hand, clustering of CD82 in MDA-MB-231 breast cancer cells attached to collagen was shown to enhance tyrosine phosphorylation of FAK (Berditchevski and Odintsova, 1999). When we replicated this assay in PC3 cells expressing CD82, we did not observe any enhancement of c-Met, p130Cas, Src, or FAK activation (not shown). Enhancement of Src or FAK activity was also not observed in CD82-transfected DU145 cells upon integrin engagement (Zhang *et al*., 2003a).

Both c-Met and Src are required for invasion and CD82 blocks c-Met, Src, and invasion. These data indicate that loss of CD82 likely increases signaling through c-Met and Src to promote increased invasion and metastasis *in vivo*. This has important implications for therapeutic targeting. Replacement of genes to restore expression is much more difficult than inhibiting the activity of active genes. Therefore, these findings indicate that targeting of c-Met and/or Src therapeutically could result in reduced metastasis by suppressing the same pathway that KAI1/CD82 normally acts to suppress.

Materials and methods

Antibodies

Antibodies against c-Met for immunoprecipitation (Met-D1) and for surface expression (Met-3) were obtained from Dr Brian Cao at the monoclonal antibody core facility at the Van Andel Institute (Hay *et al*., 2003). TS82b, antibody to CD82 used for immunoblotting, was a generous gift from Dr Eric Rubinstein (INSERM, Villejiuf, France). The CD82 antibody used for immunostaining and FACS (BD-CD82) and anti-α6 integrin antibodies were purchased from BD-Pharmingen. The monoclonal anti-Src antibody 327 has been described previously (Lipsich *et al*., 1983). The anti-phosphotyrosine monoclonal antibody 4G10 was provided by Tom Roberts (Dana Faber Cancer Institute, Boston, MA, USA). Phospho-specific antibodies against Erk1/2 (T202/Y204) and Akt (S473) were from Cell Signaling (NEN). Total Erk antibody was from Transduction Labs and total Akt antibody was described previously (Bill *et al*., 2004). Anti-phosphotyrosine antibodies specific to Src pY^{418}, FAK pY^{397}, and FAK pY^{861} were from Biosource International Inc. c-Met antibodies for immunostaining and FAK antibodies used for immunoprecipitation were from Santa Cruz Biotechnology and antibodies to the C-terminal end of FAK (FRNK) has been described elsewhere (Bill *et al*., 2004). Antibodies to p130Cas were purchased from BD Transduction labs. Anti-integrin antibodies α3 and β4 were from Chemicon, and antibodies to β1 (AIIB2) were from the Iowa Hybridoma Bank (Ames, IA).

Cell culture and transfections

Primary PEC were obtained from Dr Beatrice Knudsen, Fred Hutchinson Cancer Research Center, and cultured as previously described (Gmyrek, 2001). PC3 and DU145 cells were obtained from American Type Culture Collection (ATCC). PC3 cells were maintained in F12K medium (Invitrogen) and DU145 cells were maintained in DMEM (Invitrogen). Both media were supplemented with 10% fetal bovine serum (Gibco), 2 mM glutamine, and 50 U of penicillin and 50 μg of streptomycin/ml. PC3 or DU145 cells were transfected with a plasmid construct pCDNA3.1(PAL)N-flag.CD82, obtained from Dr Kurt Cannon, Yale University (Cannon and Cresswell, 2001). DU145 or PC3 cells at 10^6 cells per 10-cm diameter plate were transfected with 10 μg of total DNA (1 μg of CD82 or 1 μg of vector plus 9 μg pBluescript DNA) using LT1 lipid as described by the manufacturer (Pan-Vera). At 6 h following transfection, the culture media were changed to regular F12K medium with supplements and replaced 48 h later with the same culture medium containing puromycin (2 μg/ml). Puromycin-resistant clones were picked 10 days later and screened for CD82 expression by immunoblotting with TS82b antibodies. CD82-expressing clones were maintained in

growth medium containing puromycin and used in assays described below. DU145 or CD82-expressing DU145 cells were transfected as above with 1 μg of plasmid pCMVc-hMet plasmid (Rong *et al*., 1992), 1 μg pSV-neo plasmid, and 8 μg pBluescript DNA. Cells were selected in 1 mg/ml G418, and neomycin-resistant clones were selected and screened for both CD82 and c-Met expression. For ligand stimulation of c-Met, cells were serum starved overnight and then treated for 5–10 min with increasing concentrations of HGF/SF (Calbiochem) prior to lysis.

Adhesion to matrix

Adhesion to extracellular matrices was routinely performed as described by Miranti (2002). Briefly, cells were serum starved 18–24 h, trypsinized, and placed in suspension for 30 min. Cells were placed on plates coated with 10 μg/ml of Collagen I (rat tail, BD Biosciences), Laminin (mouse, Invitrogen), or Fibronectin (BD Biosciences). A suspension control was maintained at 37°C. After 45–60 min when the cells begin to adhere and spread, the cells were subjected to lysis with RIPA buffer (10 mM Tris (pH 7.2), 158 mM NaCl, 1 mM EDTA, 0.1% sodium dodecyl sulfate (SDS), 1% sodium deoxycholate, 1% Triton X-100, 1 mM Na_3VO_4, 1 mM phenylmethyl sulfonyl fluoride (PMSF), 5 μg of aprotinin/ml, 5 μg of pepstatin/ml, 5 μg of leupeptin/ml, and 1 μM benzamide). Suspension samples were subjected to lysis at the same time. After lysis, adherent and suspension samples were passed through a 27 gauge needle. All lysed samples were subjected to centrifugation at 13 000 *g* for 10 min.

For Src inhibition studies, PC3 cells were pretreated with DMSO or 2 μM SU6656 (Calbiochem) during the last 6 h of the 24 h starvation phase and maintained during adhesion to matrices.

Adhesion assay

Vector and CD82-expressing PC3 cells were starved and placed in suspension as outlined above. 96-well plates were coated with serial dilutions of laminin in triplicate from 10 to 0.078 μg/ml. Cells were allowed to adhere for 45 min. Nonadherent cells were removed by washing with PBS and cells were fixed and stained with 0.09% crystal violet stain (Chemicon) for 10 min. Alternatively, cells were allowed to adhere to 96 wells coated with 10 μg/ml laminin for various times, ranging from 10 min to 1 h. The number of cells in two random microscopic fields per well were photographed and counted.

Immunoprecipitation and immunoblotting

Protein concentrations of the samples were determined using the bicinchoninic acid assay (Pierce). Immunoprecipitation mixtures containing 500–1000 μg protein were incubated with the appropriate antibodies for 3 h at 4°C with either protein A- or protein G-conjugated agarose beads (Pierce) to capture the complexes. All immunoprecipitated complexes were washed three times with their respective lysis buffer. Immunoprecipitated samples from adhesion assays were resuspended in 2 × SDS sample buffer, boiled for 5–15 min. For immunoblotting of CD82 in cell lysates, 50 μg of protein was resuspended in 2 × sample buffer under nonreducing conditions. All resuspended samples were then subjected to SDS–polyacrylamide gel electrophoresis, transferred to a polyvinylidene difluoride membrane (PVDF). The PVDF membranes were blocked with 5% bovine serum albumin in Tris-buffered saline containing 0.1% Tween 20 for 2 h, followed by 2 h incubation with the appropriate primary antibodies. After several washes, blots were incubated with a horseradish peroxidase-conjugated secondary antibody (Bio-Rad) for 1 h and visualized with a chemiluminescence reagent (NEN). Blots were stripped in low-pH 2% SDS at 65°C for 60 min, rinsed, and reprobed for total levels of protein in the immunoprecipitates or cell lysates.

Migration and invasion assays

For migration assays, the underside of the membrane of the upper chamber of 8 μm transwell migration chambers (Corning) were coated with 10 μg/ml of collagen I, laminin, or fibronectin. Approximately 50 000–70 000 serum-starved cells of the vector- or the CD82-transfected PC3 cells were added to the upper chamber. For Src inhibition studies, cells were pretreated with DMSO or 2.5 μM SU6656 (Calbiochem) during the 18–24 h starvation phase. The cells were incubated at 37°C, and after 3 h, the cells were washed, then fixed and stained with 0.09% crystal violet stain (Chemicon) for 10 min. Cells remaining on the upper side of the membrane were removed with a cotton swab. Stained cells that had migrated through the membrane to the underside were visualized on a Nikon TE300 microscope and pictures taken using a CCD camera (Hammamatsu) attached to an Apple Macintosh G4. Images were compiled using OpenLab software (Improvision) Pictures were collected from three different fields of each chamber and counted. Invasion assays were performed on a matrigel invasion chambers (BD Biocoat, Beckton Dickinson Labware). The invasion assays were set up identically to the migration assays except that cells were fixed and stained after 72 h. Pictures of the invaded cells were taken using Nikon TE300 microscope attached to a camera as described above. For both migration and invasion assays, the number of cells in each of three fields for each assay were counted and the standard error was calculated and plotted.

Antibody crosslinking

Cells were plated on laminin, and after 1 h unattached cells were washed with DMEM. Then, 25 μg/ml mouse IgG (Pierce) or 25 μg/ml of TS82b antibodies was added. Cells were incubated at 37°C for 2 h, washed with DMEM and incubated with secondary rabbit anti-mouse antibodies at 2.5 μg/ml (Pierce) for 1 h. Cells were washed and lysed in RIPA.

Immunofluorescent staining

Immunofluorescent staining protocols were performed similar to Lee *et al*. (2004a) with some modifications. Round cover slips were coated with Collagen I or laminin (10 μg/ml, final concentration) overnight in a six-well dish. Matrix-coated cover slips were blocked with 1% BSA in PBS. Following a standard adhesion protocol (Miranti, 2002), around 50 000 cells of the vector- or CD82-transfected PC3 cells were plated on the cover slips. Adherent cells were fixed with 4% paraformaldehyde in PBS for 10 min and permeabilized with 0.2% Triton-X for 3 min. Nonspecific binding was blocked by incubating with 1% normal goat serum for 2 h. c-Met was detected with anti-c-Met antibodies from Santa Cruz Biotechnology and CD82 was detected with anti-CD82 antibodies (BD-Pharmingen). Secondary antibodies were either Alexa488-conjugated (green) or Alexa568-conjugated (red) anti-mouse secondary antibodies (Molecular Probes). Cells were counterstained with Hoechst 33258 (at 10 μg/ml) for visualization of the nuclei and mounted on a slide with 10 μl of p-Phenylenediamine PPD, an antifade agent (Sigma). Stained cells were visualized either on a Nikon TE300 microscope equipped for epi-fluorescence using a CCD camera (Hammamatsu) attached to an Apple Macintosh G4 and OpenLab software (Improvision) or with a Zeiss LSM510 Confocal Scope and Zeiss LSM Imaging Software.

Surface expression of CD82, integrins, and c-Met were analysed using a FACS Caliber (Becton Dickinson). Approximately 1×10^6 cells were collected by trypsinization and stained with the appropriate primary antibody (BD-CD82, integrins $\alpha 3$, $\alpha 6$, or $\beta 1$, or Met-3). After staining with FITC-conjugated secondary antibodies, the samples were analysed for surface expression by FACS Caliber. The level of surface expression was normalized to that observed with cells treated with IgG control antibodies. The results were generated using FACS analysis software.

Surface biotinylation

Cells were plated on laminin or were left attached to culture plates after serum starvation. Unattached cells were washed away and cells were placed on ice. EZ-link NHC-LC Biotin (Pierce, Rockford, IL, USA) at 0.5 mg/ml in PBS was added to the adherent cells and incubated at 4°C for 2 h. Cells were lysed with 1× RIPA and $\alpha 6$ integrin was immunoprecipitated. Biotin labeling was detected by immunoblotting with extravidin–peroxidase conjugate (Sigma).

shRNA inhibition of c-Met

Generation of the adenoviral vectors and virus for the expression of human c-Met shRNAs has been previously described (Shinomiya *et al*., 2004). $1–2 \times 10^6$ cells were infected at an m.o.i. of 100 with mU6-Ad1 control virus, si-hMet-Ad^{221} (Ad9), or si-mMet-Ad^{178} (Ad5). The targeted sequence for hMet-Ad^{221} is human 5′-GTGCAGTATCCTCTGACAG-3′ and for mMet-Ad^{178} is mouse 5′-GTGATCGTTCAACCG GATC-3′. The si-mMet-Ad^{178} virus was designed to inhibit mouse c-Met, but shares homology with 16 of 19 bases to human c-Met and was efficient at inhibiting human c-Met expression in both DU145 and PC3 cells (data not shown). After 2–3 days, cells were used in adhesion or invasion assays. The levels of c-Met expression in each assay were monitored by immunoblotting.

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The Impact of Cell Adhesion Changes on Proliferation and Survival During Prostate Cancer Development and Progression

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Abstract In the normal prostate epithelium, androgen receptor (AR) negative basal epithelial cells adhere to the substratum, while AR expressing secretory cells lose substratum adhesion. In contrast, prostate cancer cells both express AR and adhere to a tumor basement membrane. In this review, we describe the differential expression of integrins, growth factor receptors (GFRs), and AR in normal and cancerous epithelium. In addition, we discuss how signals from integrins, GFRs, and AR are integrated to regulate the proliferation and survival of normal and malignant prostate epithelial cells. While cell adhesion is likely of great importance when considering therapeutic approaches for treatment of metastatic prostate cancer, no data on integrin expression are available from tissues of prostate cancer metastasis. However, several drug targets that are upregulated after androgen ablative therapy regulate cell adhesion and thus novel targeted therapies indirectly interfere with cell adhesion mechanisms in prostate cancer cells. J. Cell. Biochem. 99: 345–361, 2006.

Key words: androgen receptor; integrins; prostate cancer; growth factor receptors; signal transduction

INTERACTIONS BETWEEN SUBSTRATUM ADHESION, PARACRINE GROWTH FACTORS, AND ANDROGEN IN NORMAL PROSTATE EPITHELIUM

In the human and mouse adult prostate epithelium, p63 expressing basal cells differentiate into secretory cells, transitioning through an intermediate/transiently proliferating cellular compartment. During differentiation the cells change their cytokeratin expression. Basal cells are K14 and weakly K5 positive, intermediate cells are K5 and K18 positive, and secretory cells are K18 and K8 positive. Differentiation is also accompanied by the formation of a suprabasally located secretory cell layer, loss in adhesion to the substratum, and gain in expression of the androgen receptor (AR). Thus, in normal epithelium, there is an inverse relationship between cell adhesion and AR expression.

Basal Epithelial Compartment

During prostate development androgen-regulated stromal factors, named andromedins, interact with non-androgen-regulated growth factors to stimulate epithelial morphogenesis. In contrast to basal epithelial cells, stromal cells express AR and thus proliferation, survival, and branching morphogenesis of basal epithelial cells are indirectly regulated by androgens through the prostate stroma. The responsible stromal factors include FGF7, FGF10, IGF, and HGF [Thomson, 2001; Donjacour et al., 2003; Knudsen and Edlund, 2004]. In particular, forced expression of FGF7 in the prostate stroma of transgenic mice caused epithelial hyperplasia, [Foster et al., 2002]. A recent mouse model demonstrated that stromal growth factors are also regulated via an autocrine loop that involves TGF-β in the prostate stroma

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[Bhowmick et al., 2004]. Knockout of the TGF-β type II receptor resulted in increased HGF secretion by stromal cells and the development of intraductal carcinoma.

Integrins comprise a family of cell adhesion receptors that regulate the attachment of epithelial cells to the basement membrane, also called substratum. They function as co-receptors of GFRs, allowing effective transduction of signals from the basal cell surface to the cytoplasm and nucleus. Thus, integrins are involved through multiple distinct pathways and networks in the regulation of prostate epithelial growth and oncogenesis. Here we examine integrin-mediated pathways that are specific to normal prostate basal epithelial cells and to prostate cancer cells, since these are the only two cell types that adhere to the substratum.

In the normal human prostate gland, AR-deficient basal cells adhere to substratum containing collagen IV, collagen VII, laminin 5, and laminin 10/11 [Knox et al., 1994]. Adhesion to collagen IV is mediated by integrin α2β1, while adhesion to collagen VII and laminin 5 is mediated through α6β4 and α3β1. Studies in α3, α6, β4 or laminin 5 null mice suggest a high level of redundancy of these components for basal cell function [Ryan et al., 1999; DiPersio et al., 2000]. All of these mice develop a severe blistering phenotype in the skin and oral epithelium following birth, likely caused by abrasive action. However, the normal architecture of stratified epithelial differentiation in the skin is maintained prior to birth. Extensive apoptotic cell death, termed anoikis, occurs in blisters where epithelial cells detach from the substratum, clearly demonstrating the important role of substratum adhesion for cell survival of basal cells. In contrast to laminin 5 null mice, collagen IV deficient mice fail to stabilize basement membranes [Poschl et al., 2004]. Surprisingly, integrin-α2 null mice do not develop a blister phenotype [Chen et al., 2002]. In general, the prostates of viable integrin null mice at birth were not examined and since knockouts cause neonatal lethality, tissue transplantation is necessary to determine the role of integrins during branching morphogenesis and epithelial differentiation of the prostate.

Attachment of quiescent epithelial cells to the substratum occurs primarily via integrins α6β4 in hemidesmosomes. The crosstalk between hemidesmosomes, which anchor cells to the substratum and E-cadherin-based cell–cell interactions helps to limit proliferation. During cell division there is a temporary disruption of cell–cell and α6β4 hemidesmosomal interactions and this removes the brake that acts to suppress growth and migration of cells. When α6β4 interactions are disrupted, engagement of α3β1 integrins may increase temporarily to support cell proliferation. The α3β1 integrins are typically localized within the basal-lateral cell membrane and may not be fully engaged through substratum binding in non-proliferating basal cells [Yanez-Mo et al., 2001]. Thus, integrin utilization and not just integrin expression levels determine interactions of cells with the substratum and regulate cell proliferation and migration.

Intermediate/Transiently Proliferating Cell Compartment

Prostate basal cells are the first epithelial cell type in the prostate to appear during development and are responsible for ductal morphogenesis. We now appreciate that basal cells differentiate into intermediate cells. The intermediate compartment is divided between the basal and suprabasal/secretory cell layers [van Leenders et al., 2003; Uzgare and Isaacs, 2004]. When visualized by staining with Ki-67/MIB1, proliferative cells are observed along the basement membrane and daughter cells move into the suprabasal layer. During this transition, cells experience the greatest change in integrin expression. As cells lose substratum adhesion, integrin expression diminishes. Most notably is the loss in β4 integrin expression, resulting in an increase in α6β1 [Cress et al., 1995]. There is also a concomitant decrease in expression of the other β1 integrins, α3β1 and α2β1. A similar loss in β4 integrin expression occurs during keratinocyte differentiation, which can be triggered by PKCδ activation or Myc expression [Gandarillas and Watt, 1997; Alt et al., 2001; Gebhardt et al., 2006]. It is uncertain whether the loss of integrin expression is caused by the gain in AR expression, since in prostate cancer cell lines forced expression of AR reduces expression of β4 and other integrins [Bonaccorsi et al., 2000; Nagakawa et al., 2004]. Thus, it is conceivable that adhesion to basement membrane exerts a negative regulatory effect on AR expression and that the loss of cell adhesion is a requirement for AR protein expression in the normal prostate epithelium.

The intermediate compartment expands in atrophic glands and in a common condition by the name of proliferative inflammatory atrophy (PIA). Characteristically in PIA, intermediate cells in the suprabasal layer label with Ki-67/MIB1 [van Leenders et al., 2003]. However, it is unclear whether the Ki-67 positivity reflects G0/G1 and G1/S phase progression in the suprabasal layer, or whether cell-cycle entry is initiated in the basal layer and cells move suprabasally during cytokinesis in G2/M maintaining Ki-67 expression. It is conceivable that because these cells do not "sense" a substratum, they fail to re-express integrins in the subrabasal layer after cell division. In the suprabasal layer, intermediate cells express a low level of nuclear AR. We postulate that in suprabasal intermediate cells, androgen stimulates differentiation and not proliferation, since as AR expression increases, proliferation declines. Thus, androgen is not the driving force for expansion of this compartment in PIA. This is consistent with the inhibition of cell growth by androgen in AR-expressing cultured primary epithelial cells, which are of the basal and intermediate phenotype [Robinson et al., 1998; Fry et al., 2000; Lang et al., 2001; Berger et al., 2004]. Histologic inspection and immunohistochemical visualization of AR protein in normal prostate epithelium clearly demonstrates that adhesion to the substratum and expression of AR occurs in distinct cell types and distinct epithelial cell layers. While cell adhesion receptors regulate the proliferation of intermediate cells that are attached to the substratum, androgen and AR likely mediate growth arrest and differentiation in suprabasal intermediate cells as they begin to further differentiate into secretory cells.

Secretory Epithelial Compartment

The differentiated secretory cells in the mouse and human prostate are terminally differentiated and post-mitotic, and thus in these cells AR does not stimulate cell proliferation but regulates the synthesis and secretion of proteins. The effects of androgen on epithelial homeostasis have been primarily explored in rodent prostate. Enforced hyper-expression of AR in otherwise normal mouse secretory epithelium does not stimulate proliferation suggesting androgen action is likely necessary for cell viability and differentiation [Han et al., 2005]. Indeed, in adult mice, castration leads to massive apoptosis of secretory epithelial cells within 48–72 h. Secretory epithelial apoptosis in castrated mice is attributed to the decline of paracrine stromal factors. While the mouse epithelium contains sparse basal cells, the human gland is lined by a continuous basal cell layer, a barrier that potentially shields secretory cells from stromal factors. Thus in mice, androgen-regulated stromal factors have easy access to secretory cells, whereas this is not the case in the human gland. Because of this difference stromal-epithelial interactions may not be the same in mouse and human prostate. In fact, careful inspection along prostatic ducts in rodent prostate revealed less apoptosis in the proximal, basal cell-rich region compared to the tips of the ducts. Based on this observation a barrier function of the basal epithelial layer was suggested [Tenniswood et al., 1992].

Upon androgen suppression in patients, secretory cell numbers diminish. However, focal areas of viable epithelium may persist even after 6–9 months of treatment, indicating that the prostate tissue can maintain an elevated androgen level even when serum androgen is markedly decreased, or that androgen may not directly support the viability of secretory epithelial cells. It is unclear whether secretory cells die because of a change in paracrine factors from the stroma or because of loss of intrinsic AR activity.

In summary (Table I), in the normal human adult prostate epithelium cell adhesion to the substratum and expression of AR occur separately in the basal and suprabasal-luminal cell layers, respectively. Thus, in normal epithelium, signaling pathways from cell adhesion and androgen stimulation do not interact. While adhesion to the substratum facilitates the transduction of stromal signals and mediates cell proliferation and survival, androgen primarily causes protein secretion and might maintain the viability of luminal prostate epithelial cells.

CHANGES IN SUBSTRATUM ADHESION, GFR EXPRESSION, AND ANDROGEN RESPONSIVENESS DURING PROSTATE CANCER DEVELOPMENT

Tissue Analysis of Prostate Cancer Development and Androgen Responsiveness

The development of invasive prostate cancer occurs through an intermediary in situ carcinoma stage, which is referred to as prostatic intra-epithelial neoplasia (PIN). In the early stage of PIN, AR expressing carcinoma cells

reside above a continuous basal cell layer. As PIN progresses, the basal cells disappear and carcinoma cells adhere directly to the substratum (see Table I). Theories for the loss of basal cells include overgrowth of carcinoma cells, invasion of carcinoma cells into the basal cell layer, and apoptosis of basal cells [Bonkhoff, 1996; Yu et al., 2004]. As prostate cancer invades, AR expressing tumor cells interact through integrins with the substratum. Coincidently, the androgen-axis stimulates cell proliferation and survival, in addition to protein secretion. Therefore, de novo adhesion of prostate cancer cells to the substratum may regulate the activity of the AR in prostate cancer cells. This is strikingly different to normal epithelium where substratum adhesion and growth factor activation are spatially separated from AR expression into two different cell layers. Thus, we propose that the switch in AR function to promote proliferation and survival in cancer cells, as opposed to growth suppression and differentiation in normal cells, is facilitated by the interaction of cancer cells with the substratum and the integration of downstream signaling pathways from integrins, growth factors receptors, and AR. We will present examples in a later section to illustrate how cancer cells integrate the downstream pathways from these three signals.

Androgens have a marked effect on prostate cancer cell proliferation and viability in vivo. When patients with androgen-sensitive metastatic disease are androgen ablated the proliferation of cancer cells in the prostate 5 significantly inhibited and massive numbers of cancer cells eventually die. In addition, the proliferation of cancer cells in the prostate is significantly inhibited by anti-androgenic therapy and cancer cells eventually die [Reuter, 1997]. During prolonged androgen suppression, the cytoplasm of cancer cells and of normal secretory cells becomes vacuolated and the nuclei are irregular and mildly pyknotic. These histological features, in addition to the long duration before cell death, are suggestive of autophagy and not of apoptosis. Thus, in contrast to basal cells, adhesion to the substratum is not sufficient for survival of androgen-dependent prostate cancer cells and in the absence of androgens, cancer cells stop proliferating and eventually die. However, an androgen independent population of cancer cells may arise, whose survival is no longer dependent on androgen. This likely occurs through the acquisition of additional oncogenic events that reduce the androgen requirement for activation of AR. It is likely that integrins play an important role in the progression to androgen-independent disease because they augment the activity of kinases that phosphorylate and activate the AR under reduced androgen concentrations. The emerging cells may have a greater dependence on cell adhesion to the substratum for survival, compared to androgen-dependent tumors, and use the substratum to regulate GFR and AR signaling to enhance cell survival.

Integrins and Extracellular Matrix Proteins in Locally Invasive Prostate Cancer

Invasive prostate cancer glands in humans are lined by a single layer of tumor cells. The cancer cells retain certain properties of basal cells, but also express markers of secretory cells including cytokeratin 8 and 18, AR, and PSA. The observation that cancer cells are differentiated according to cytokeratin 8 and 18 expression and positivity for AR and PSA, but negative for basal cell markers, p63, keratin 5 or 14. The observation that cancer cells coexpress basal and secretory cell markers prompted a model in which oncogenic transformation occurs within the intermediate compartment and triggers an aberrant differentiation program. As a result, we would expect that GFRs normally expressed on basal and not on secretory cells remain expressed in some cancer cells. Indeed, several GFRs are noticeably elevated in prostate cancer cells compared to secretory cells [Ware, 1998; Knudsen et al., 2002].

However, because cancer cells are more differentiated than basal cells and because cancerous glands lack a basal cell layer, we anticipate differences in cell adhesion complexes as well as substratum constituents between normal epithelium and cancer. Our insight into integrin expression and substratum composition is based on a detailed immunohistochemical analysis in frozen tissues [Knox et al., 1994; Cress et al., 1995]. The substratum of tumor glands, compared to normal glands, is altered. Specifically, laminin 5 and collagen VII are lost in cancer, but laminin 10/11 and collagen IV are retained. This alteration directly correlates with the loss of the laminin 5 binding integrins, $\alpha 6\beta 4$, and the reduced expression of $\alpha 3\beta 1$ on cancer cells. The prostate cancer integrin, $\alpha 6\beta 1$, engages laminin 10/11 [Cress et al., 1995]. Compared to the normal epithelium, two addi-

tional differences in integrin expression exist in the carcinoma cells: the β1C integrin splice-variant shifts to β1A and a truncated α6 variant, α6p, is abundantly expressed [Fornaro et al., 2000; Demetriou et al., 2004]. The changes in integrin and substratum protein expression are likely to be important in tumor development. For instance integrin β1A stimulates proliferation in vitro [Goel et al., 2005], while α6p integrin triggers invasion [Rabinovitz et al., 1995]. Thus, signaling specifically through laminin 10/11 and an α6(p)β1A integrin variant may enhance tumorigenesis. In addition it is likely that intracellular changes in signal transduction pathways accompany α6(p)β1A expression. Whether or not these affect AR function remains to be investigated.

The α6p variant may also be important in the development of metastatic cancer. α6p is generated by cleavage of its extracellular domain by the extracellular protease uPAR. It lacks the ligand-binding domain and therefore no longer interacts with the substratum [Demetriou et al., 2004]. The result would be decreased adhesion and increased mobility in tissues [Blasi and Carmeliet, 2002]. Interestingly, the tetraspanin CD82, which is an α6 integrin-interacting protein and a metastasis suppressor gene, suppresses uPAR activity [Bass et al., 2005]. Thus, loss of CD82 expression during tumor progression may be one of the reasons for increased uPAR activity and cleavage of α6 integrin.

AR, Integrins, and GFRs in Locally Invasive Prostate Cancer

When expressed in basal epithelial cultures or in the PC3 cancer cell line, AR suppresses cell proliferation, while in xenografts of most prostate cancer cell lines, and in prostate cancer in vivo, androgen stimulates cell proliferation [Heisler et al., 1997; Berger et al., 2004]. Therefore, there may be a disconnect between AR and androgen action. In transgenic mice, a single point mutation in AR was sufficient to trigger tumor development and progression while enforced hyper-expression of wild-type AR was not [Han et al., 2005]. On the other hand, oncogenic immortalization of normal human prostate epithelial cells and co-expression of wild-type AR was sufficient to induce androgen-dependent tumors in a xenograft model [Berger et al., 2004]. In these tumors androgen was necessary for cell proliferation. The mutationally activated AR alone is sufficient to induce prostate cancer in mice; however, in human cancer AR mutations occur late in oncogenesis and are not the cause for prostate cancer development. Together these results suggest that the proliferative activity of the AR is context dependent and requires oncogenic transformation. It is likely that oncogene-induced enhanced expression and activation of GFRs and integrins are required to increase the proliferative activity of the AR.

Evidence for cooperation between integrins and GFRs for regulating cell proliferation has been well documented [Miranti and Brugge, 2002]. Integrin crosstalk with IGFR1 is required for efficient IGF-1 signaling [Walker et al., 2002; Clemmons and Maile, 2005]. In return, IGF-1 enhances integrin-mediated adhesion and spreading [Hermanto et al., 2002]. Recent studies have demonstrated that IGFR is critical for prostate cancer development [Wu et al., 2005a] and transgenic mice expressing high levels of IGF-1 under control of the K5 promoter develop prostate tumors [DiGiovanni et al., 2000]. IGFR1 forms complexes with α6 integrin and recruits activated MAPK [Walker et al., 2002]. Thus, integrin α6β1-laminin 10/11 interactions may cooperate with IGF/IGFR to promote the early development of prostate cancer. Interestingly, the β1A integrin variant, which is increased in prostate cancer, promotes IGF-1-mediated cell proliferation, while the β1C integrin variant, which is downregulated in prostate cancer, inhibits IGF-mediate proliferation [Goel et al., 2005]. A monoclonal antibody to IGFR1 inhibits tumor proliferation in both AR-dependent and AR-independent models of LUCap35 prostate cancer xenografts, suggesting that androgen and IGFR independently support proliferation [Wu et al., 2005a]. Future research will need to address which oncogenic events cause a switch in AR activity from inhibition to stimulation of cell proliferation, and determine whether cancer specific cell adhesion to basement membrane or expression of GFRs contribute to the switch in AR activity.

AR, INTEGRINS, AND GFRS IN THE REGULATION OF CELL PROLIFERATION AND SURVIVAL IN METASTATIC PROSTATE CANCER

Integrins and Extracellular Matrix in Metastatic Prostate Cancer

Understanding of the role of integrins in prostate cancer metastasis has been stymied

by the lack of in vivo immunohistochemical data in metastatic tissues and thus our current knowledge is derived from analyzing cell lines [Fornaro et al., 2001]. Cellular models of prostate cancer progression have largely been derived from two cell lines, LNCaP and PC3. In these models integrin expression, utilization, and function have been studied; however, it is uncertain whether in vivo correlates exist. The LNCaP cells are androgen-sensitive and thus recapitulate characteristics of androgen-responsive primary prostate cancer cells. One might therefore expect that LnCaP cells express a repertoire of integrins that is similar to primary prostate cancer. However, contrary to primary prostate cancer tissues, LNCaP cells express low levels of the laminin receptors α6β1 and α3β1, but high levels of the fibronectin receptor, α5β1 [Witkowski et al., 1993; Edlund et al., 2001]. PC3 and DU145 cells, derived from metastatic lesions, do not express AR and express elevated levels of α5β1, as well as the vitronectin receptor, αvβ3, neither of which have been reported to be expressed in vivo [Cooper et al., 2002]. αvβ3 expression is typically not seen in normal epithelial cells, although one report suggests that it is expressed in primary tumors [Zheng et al., 1999]. Whether α5β1 or αvβ3 are expressed in metastatic tumors in vivo has not been demonstrated. In cell lines, expression of integrins α5β1 and αvβ3 might be caused by high fibronectin and vitronectin levels in serum used for cell culture. The selection pressure during the establishment of cells lines could favor cells that upregulate fibronectin- and vitronectin-binding integrins. Changes in integrin expression and function during prostate cancer progression and metastasis formation might be an important contributing factor to tumor growth and development of treatment resistance.

The role of β4 integrin in metastatic prostate cancer remains controversial. Elevated levels of β4 integrin have been routinely observed in primary and metastatic breast and colon cancers [Natali et al., 1992; Davis et al., 2001]. However, it is not expressed in primary prostate cancers in vivo. Metastatic prostate cancer cell lines express β4 integrin, but it is not known whether β4 is expressed in prostate metastases in vivo. Since forced expression of AR in metastatic cell lines decreases expression of β4 integrin, β4 expression in cell lines may simply indicate the loss of the androgen/AR signaling axis due to in vitro culturing [Bonaccorsi et al., 2000; Evangelou et al., 2002; Nagakawa et al., 2004]. However, β4 integrin expression in metastatic cells could serve a different function than in basal epithelial cells, since metastatic cells do not form hemidesmosomal structures and fail to deposit laminin 5. If β4 integrin expression in metastatic prostate cancer cell lines recapitulates integrin expression in prostate cancer metastasis in vivo and is not an artifact of cell culture, then re-expression of β4 in androgen-independent tumors may play a unique role in prostate cancer metastasis. Further studies will be necessary to validate the role of β4 integrin in metastasis.

In addition to the reported changes in substratum in primary prostate cancer, it is expected that metastatic cells will see an even different substratum. Over 80% of prostate metastases are found in the bone. Collagen I is one of the primary substratum proteins in the bone and α2β1 integrin is primarily responsible for adhesion to collagens. While basal prostate epithelial cells express α2β1 and bind collagen IV in the basement membrane [Knox et al., 1994], there is significantly less, but measurable expression of α2β1 in primary prostate cancers. The metastatic cell lines express α2β1 at levels similar to basal cells, with PC3 cells showing slightly higher levels of expression. Treatment of PC3 cells with the bone-derived growth factor, TGF-β1 increases α2β1 levels as well as adhesion and spreading [Kostenuik et al., 1997]. Thus, signaling through collagen/TGF-β1 in the bone environment may favor metastatic growth in part through increasing integrin engagement. How signaling through TGFβ1 and α2β1 impacts AR function in the metastatic cells is unknown.

Integrin expression and function can be modulated by interactions with other proteins, which may be important in metastasis. CD82/KAI1 was identified as a metastasis suppressor of prostate cancer cells in a metastasis screen in rats [Dong et al., 1996]. CD82 is a tetraspanin that controls the activity of the prostate cancer integrins, α6β1 and α3β1 [Maecker et al., 1997] as well as negatively regulates EGFR and c-Met [Jackson et al., 2003; Odintsova et al., 2003; Sridhar and Miranti, 2005]. CD82 exerts its effects by limiting the distribution and association of integrins and GFRs on the cell surface. Thus, in advanced stages of prostate cancer reduced expression of CD82 as it is documented

to occur in vivo would permit associations and redistribution of integrins and GFRs leading to enhanced signaling and an augmentation of cell proliferation and androgen insensitivity.

Crosstalk Between Integrins, AR, and GFRs in Metastatic Prostate Cancer

AR is considered the main culprit of metastatic growth and treatment failure [Grossmann et al., 2001]. Although metastatic tumors are androgen independent, they still rely heavily on AR for growth and survival [Feldman and Feldman, 2001]. Signaling pathways emanating from GFRs and integrins may reduce the dependence on androgen and augment the transcriptional activity of AR in the progression to androgen independent disease. From studies in cell culture models, several mechanisms for interactions between integrins, GFRs, and AR have been reported and can be grouped into four paradigms.

Paradigm 1: Signal transduction pathways from integrins and GFRs activate kinases that affect the expression and activity of AR and AR-coregulators through phosphorylation. Enhanced signaling through GFRs is thought to play an important role in enhancing AR activity, especially in the progression to androgen independence. The crosstalk between integrins and GFRs also intensifies during prostate cancer progression particularly if GFRs are over expressed. For example, at normal expression levels, engagement of integrins activates the EGFR kinase, but this is not sufficient to induce cell proliferation. However, over expression of EGFR, permits cell-cycle progression through integrin engagement [Bill et al., 2004]. Several studies have attempted to address whether members of the EGFR family, including ErbB2/Her2/Neu, are significantly over expressed in prostate cancer metastases. So far there is limited evidence to support ubiquitous over expression [Ware, 1998]. In contrast to the EGFR family, c-Met is expressed in practically all metastatic prostate cancers and is significantly over expressed in prostate bone metastases compared to soft tissue metastasis [Knudsen et al., 2002]. Its loss in PC3 cells results in apoptotic cell death [Shinomiya et al., 2004] and c-Met could therefore be a driver of metastatic growth and tumor cell survival. A recent study demonstrated a shift from paracrine growth stimulation of the androgen-dependent CWR22 xenograft model to autocrine growth stimulation through hepatocyte growth factor (HGF) secretion in the androgen-independent xenograft [Nakashiro et al., 2004]. In patients, the c-Met ligand, HGF, is secreted by osteoblasts and thus paracrine activation of c-Met could occur in metastatic prostate cancer cells in the bone. However, if c-Met is sufficiently over expressed, activation may be integrin and not HGF dependent [Wang et al., 2001]. Finally, the observation that loss of the integrin binding protein CD82 enhances c-Met activation by both integrins and ligand and CD82 loss correlates with poor prognosis and metastatic disease further supports the potential importance of c-Met in prostate cancer metastasis [Sridhar and Miranti, 2005]. Thus, c-Met is a candidate GFR that through crosstalk with integrins might activate cytoplasmic kinases that phosphorylate AR.

Multiple cytoplasmic kinases, including PKA, PKC, and MAPK phosphorylate AR in its N-terminal domain [Alt et al., 2001; Gioeli et al., 2002] (Fig. 1B). These kinases have been reported to cause AR activation downstream of cell surface receptors for IGF-1, KGF, EGF or downstream of ErbB2, even when androgen concentrations are low [Grossmann et al., 2001; Culig et al., 2002; Chatterjee, 2003; Rahman et al., 2004]. However, it is uncertain whether kinase activation always permits a proliferative function of AR. In two separate studies increased expression of active MAPK was noted in the center of prostate cancers or in areas of increased cancer cell proliferation [Gioeli et al., 1999; Paweletz et al., 2001]. Therefore, phosphorylated MAPK can provide a signal for differentiation [Gmyrek et al., 2001] (central region) or proliferation (high grade cancer), and it is conceivable that the MAPK signal is in part propagated through phosphorylation of AR. Thus, the phosphorylated AR might stimulate differentiation or proliferation dependent on the underlying spectrum of oncogenic changes in the cancer. Based on preclinical studies in LNCaP cells demonstrating that knockdown of AR causes cell-cycle arrest, and under some conditions apoptosis, in LNCaP cells [Zegarra-Moro et al., 2002; Liao et al., 2005; Yang et al., 2005], eliminating AR protein expression as well as inhibiting the Ras/MAPK pathway are currently being considered as therapeutic approaches. However, it may be necessary to thoroughly evaluate the activity of the AR and MAPK throughout the cancer and avoid using

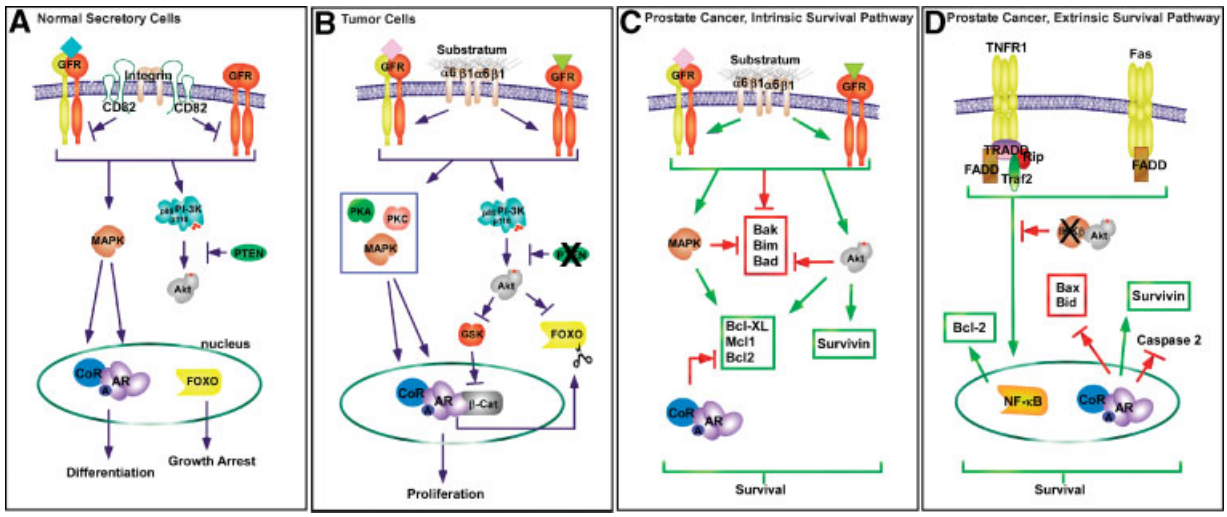


Fig. 1. Convergence of signal transduction pathways from integrins, cell surface receptors, and the androgen receptor regulate differentiation, proliferation, and survival. **Panel A**: Normal secretory epithelium. The expression of growth factor receptors (GFR) and integrins in secretory epithelial cells is low compared to basal epithelial cells or prostate cancer cells. Secretory cells do not directly contact the substratum. CD82 is expressed and limits the activity of GFRs. The Akt pathway is not significantly activated, since PTEN is present, and the FOXO proteins are in the nucleus, inhibiting cell proliferation. It is conceivable that MAPK is activated, since sustained MAPK activation stimulates cellular differentiation. In this case, active MAPK might phosphorylate the AR and AR co-regulators (CoR). The AR is in the nucleus and androgens induce and maintain a differentiation phenotype. Secretory cells are post-mitotic and it is unclear which proteins and pathways are responsible for their survival. **Panel B**: Prostate cancer cells. Prostate cancer cells express integrins α6β1 (or α3β1). The loss of CD82 permits interaction of integrins with growth factor receptors (GFR), leading to their activation and induction of signal transduction pathways. The convergence of signals from integrins and GFRs regulates cytoplasmic kinases (PKA, PKC, and MAPK), which phosphorylate AR and AR co-regulators. Phosphorylation regulates interactions between the AR and AR binding protein as well as interactions between AR and other transcription factors. The PI3K/Akt pathway plays a central role in proliferation and survival of prostate cancer cells, in part through regulating the activity of FOXO transcription factors. Akt phosphorylates FOXO proteins, sequesters them in the cytoplasm and thereby inhibits their anti-proliferative activity. FOXO proteins are cleaved by an androgen-induced protease and this may contribute to the proliferative effects of androgen in prostate cancer cells. In addition, Akt inhibits GSK3β, which stabilizes β-catenin and leads to its enhanced expression. Nuclear translocation of β-catenin may be assisted by binding to the AR and in the nucleus β-catenin stimulates cellular proliferation. **Panel C**: The intrinsic apoptotic pathway in prostate cancer cells. Integrins, growth factor receptors (GFR), and AR regulate expression of Bcl-2 and BH3 family proteins. MAPK and Akt induce expression (green) of prosurvival proteins, Bcl-2, Bcl-XL, and Mcl-1, while decreasing expression (red) of Bad, Bak, and Bim. The expression of the prosurvival protein, Survivin is also upregulated by Akt. AR suppresses transcription of Bcl-2. The balance between integrin and GFR positive signals and AR-driven negative signals determine cell fate. **Panel D**: The extrinsic apoptotic pathway in prostate cancer cells. Cell surface receptors (TNFR1, Fas) that stimulate apoptosis limit the viability of prostate cancer cells through regulation of NF-κB. Nuclear translocation of NF-κB is tightly regulated and in the nucleus where it stimulates Bcl-2 expression. Nuclear translocation of NF-κB is inhibited through binding to phosphorylated IκB. IκB is phosphorylated by IκB kinase (IKK), which is phosphorylated by Akt and targeted for degradation. Thus, Akt activation causes nuclear translocation of NF-κB. AR can inhibit apoptosis by suppressing the transcription of caspase 2 through an androgen receptor-binding element in the promoter of caspase 2.

these therapies for cancers in which AR or MAPK primarily cause cell differentiation.

Another probable mechanism for cooperation between AR, integrins, and GFRs is through transcriptional coregulators. Progression to androgen independence is associated with changes in AR coactivator expression. Coactivators enhance AR function by bridging to non-androgen regulated transcription factors and thereby connecting androgen dependent and independent pathways. While much in vitro data have linked coactivators to enhanced signaling by AR, evidence that this occurs in vivo is still lacking. The AR coactivators, ARA70, ARA55, and ARA54, are over expressed during androgen ablation and in androgen insensitive tumors [Culig et al., 2002; Chatterjee, 2003]. Over expression of either coactivator increases the sensitivity to androgens, anti-androgens, and estrogens. SRC-3 and ARA70 interactions with AR are enhanced by their phosphorylation through ErbB2/EGFR and activation of MAPK [Heinlein and Chang, 2004]. ErbB2 also stimulates PI3K (probably as a dimer with ErbB3), which leads to phosphorylation of AR on Ser213/791 [Culig et al.,

2005], however, the importance of these phosphorylation sites for AR activity is debatable. The AR coactivator, ARA55, takes a unique position, since its phosphorylation by the integrin-regulated kinase Pyk2 blocks its binding to AR [Heinlein and Chang, 2004]. Thus, the combination of coactivator and AR over expression and the regulation of interactions by integrin-activated signaling pathways may provide the underlying cause for the switch of a growth inhibitory to a growth stimulatory effect of the androgen axis during prostate cancer development.

Paradigm 2: Integrins and androgen cooperatively promote cell survival by regulating the Akt pathway and the expression of pro-survival Bcl-2 family proteins and Survivin.

Androgen/AR and regulation of the Akt pathway. The most frequently affected and best-studied survival pathway in prostate carcinoma is the PI3K/Akt pathway. In cancer cells, Akt is activated by integrins, growth factors, and through loss of the tumor suppressor, PTEN. In human samples, increased Akt activity correlates with advanced disease and high Gleason score [Ghosh et al., 2003], is an adverse prognostic indicator [Ayala et al., 2004; Kreisberg et al., 2004], and increases the danger of cancer recurrence [Thomas et al., 2004]. Conditional loss of both alleles of PTEN in AR-expressing epithelial cells is sufficient to induce prostate cancer in mice [Wang et al., 2003]. In prostate cancer cells with intact PTEN expression the Akt pathway is activated by GFRs. For instance, IGFR or ErbB2/ErbB3 mediates activation of the PI3K/Akt pathway which leads to increased activity of AR [Heinlein and Chang, 2004]. In prostate cancer cells, activation of IGFR by its ligand IGF-1 is increased through elevated IGFBP-5 and decreased through diminished IGFBP-3 expression [Culig et al., 2002]. Thus, even in tumors where PTEN is intact, signaling by growth factors could stimulate survival through PI3K/Akt.

Another mechanism for activating the Akt pathway is through integrins (Fig. 1B). Integrins are essential co-receptors for growth factor-mediated activation of the Akt pathway [Cabodi et al., 2004]; however, there is also evidence that integrins can activate the Akt pathway through GFRs, independent of growth factors [Moro et al., 1998]. Cells prevented from adhering to the substratum undergo a form of apoptotic cell death termed anoikis [Reddig and Juliano, 2005], and in some cell types adhesion-induced cell survival depends on PI3K [Frisch and Screaton, 2001]. However, PI3K/Akt is not always responsible for adhesion-mediated cell survival, and in particular adhesion of basal cells to laminin 5 does not significantly activate PI3K, neither does blocking PI3K lead to cell death [Lin et al., 1999; Uzgare and Isaacs, 2004]. In cultured basal prostate epithelial cells, cell survival on laminin 5 requires signaling from $\alpha3\beta1$ integrin via EGFR to active the Ras/MAPK pathway (Miranti, unpublished data) [Manohar et al., 2004]. Oddly, death of primary prostate epithelial cells induced by loss of $\alpha3\beta1$/laminin 5 signaling does not occur through the classical intrinsic apoptosis pathway, contrary to tumor cells which die through activation of classical apoptosis pathways [Uzgare and Isaacs, 2004]. Thus, during tumor development loss of laminin 5 and $\alpha3\beta1$, or dependence on $\alpha6\beta1$ and laminin 10/11 signaling may alter adhesion activated cell survival pathways. Changes in cell adhesion, activation of growth factors receptors, and PTEN loss may all exert selective pressures on cancer cells that affect their dependence on androgen as a survival factor.

While activation of the PI3K/Akt pathway is critical for survival, its inhibition is not sufficient to cause death of cancer cells and can be rescued by androgen or growth factors. Thus, in addition to inhibiting the PI3K pathway, the removal of androgens or growth factors is required for inducing apoptosis [Carson et al., 1999; Lin et al., 1999; Murillo et al., 2001] and both PI3K-dependent and -independent survival pathways operate in prostate cancer cells to maintain viability [Carson et al., 1999]. This has important implications for therapeutic strategies, as simply inhibiting Akt would not be cytotoxic for cancer cells and cause tumor regression.

Androgen/AR and regulation of extrinsic and intrinsic apoptotic pathways. Whether a cell lives or dies is in part determined by the activity of the extrinsic and intrinsic apoptotic pathways. While the extrinsic apoptotic pathway signals downstream of death cell surface receptors, the intrinsic pathway is regulated through expression of Bcl-2 family members (Fig. 1C,D). Both pathways interact with the androgen/AR axis. Intrinsic apoptosis is driven by Bax and Bak [Wei et al., 2001]. Bax and Bak are antagonized by three Bcl-2-family members, Bcl-2, Bcl-XL, and Mcl-1 [Gelinas and White,

2005] and increased expression of all three has been noted in mid to late stage prostate cancer [Krajewska et al., 1996]. Since Bcl-2 expression in prostate cancer is associated with tumor progression, its expression level is of keen importance and is regulated by androgen, growth factors, and integrin expression.

Upon GFR activation, the Akt pathway may be synergistic with Bcl-2 for cell survival [Huang et al., 2001]. In addition to Bcl-2, Bcl-XL may also assume an important role in supporting cell viability. In PC3 and LNCaP cells, Bcl-XL sustains survival when the PI3K pathway is inhibited [Yang et al., 2003]. There is clear antagonism between androgens and Bcl-2. Evidently in vivo, androgens suppress Bcl-2 transcription and androgen ablation upregulates Bcl-2 [Huang et al., 2004b]. Furthermore, the ability of Bcl-2 to enhance cancer growth only occurs in androgen-depleted conditions. Thus, increased Bcl-2 expression might be a requirement for progression to androgen independence [Grossmann et al., 2001]. Thus, both Bcl-2 and Bcl-XL appear to be important for survival of prostate carcinoma cells in the absence of androgens or in low androgen conditions.

In androgen-dependent cells, TNF-α activation normally induces cell death through the extrinsic cell death pathway. Surprisingly, upon removal of androgens and sustained Akt pathway activation, TNFα stimulates cell survival [Catz and Johnson, 2003]. This response requires the degradation IKK, which permits nuclear translocation of NF-κB and increased Bcl-2 transcription (Fig. 1D). Since the NF-κB pathway is suppressed by androgens, this may explain the increase in Bcl-2 expression upon androgen ablation.

Integrins stimulate cell survival through upregulation of Bcl-2 proteins and through inhibition of proapoptotic proteins such as Bim, Bad, and Bak [Zhang et al., 1995] (Fig. 1C). Studies in our lab with basal prostate epithelial cells and in other labs with keratinocytes indicate that adhesion to laminin-5 regulates cell survival through α3β1 integrin-mediated activation of the Ras/MAPK pathway [Ryan et al., 1999; DiPersio et al., 2000]. We further noted upregulation of Bcl-XL and the downregulation of Bim under the same conditions (Miranti, unpublished data). Thus, the changes in integrin expression during prostate cancer progression may regulate cell survival through Bcl-XL expression.

In vivo androgens clearly regulate tumor cell survival, however, how AR interacts with the intrinsic or extrinsic pathways is largely unknown (Fig. 1C,D). The recent discovery of a functional ARE in the caspase 2 gene and its inhibition by androgen, suggests that this may be a mechanism by which androgens directly regulate apoptosis [Rokhlin et al., 2005]. Decreased expression of caspase 2 was sufficient to prevent TNFα- or TRAIL-induced apoptosis. Androgens can also mediate cell survival by inducing expression of Survivin. Survivin is an anti-apoptotic protein that blocks caspase activity [Goel et al., 2005]. In a recent study in metastatic PC3 cells, adhesion to fibronectin was found to upregulate Survivin levels. This was dependent on PI3K and responsible for inhibiting TNFα-induced apoptosis [Fornaro et al., 2003]. Thus androgens, GFRs, and integrins all regulate many of the same molecules and pathways that are important for survival of both normal and tumor cells. The relative intensity of signals from each pathway, the presence of oncogenic mutations, and the extent of crosstalk will determine which pathways predominate and likely guide tumor progression.

Paradigm 3: AR interacts with transcription factors that are activated at the end of signal transduction pathways. One possible explanation for the interdependence of PI3K and AR in promoting cell survival is that they cooperate to reduce the activity of forkhead box-O transcription factors (FOXO). Translocation of FOXO transcription factors into the nucleus triggers growth arrest and apoptosis (Fig. 1A). Akt inhibits FOXO proteins by direct phosphorylation, which causes their sequestration in the cytoplasm [Greer and Brunet, 2005]. Under certain conditions, AR may bind FOXO1 and inhibit nuclear entry [Li et al., 2003]. In addition, androgen induces a cysteine protease that cleaves and inactivates FOXO1 [Huang et al., 2004a]. An important cell proliferation target of FOXO proteins is the Cdk2 inhibitor, p27kip [Lynch et al., 2005] whose loss of expression is associated with prostate cancer development in mice and adverse patient outcome [Di Cristofano et al., 2001].

The recent finding that β-catenin shuttles with AR into the nucleus and is found in AR transcriptional complexes, suggests that the Wnt signaling pathway interacts with AR [Song and Gelmann, 2005; Verras and Sun, 2005]. Akt phosphorylation of GSK3β further enhances

β-catenin/AR interactions by stabilizing the cytoplasmic expression of β-catenin (Fig. 1B) [Sharma et al., 2002; Mulholland et al., 2006]. Whether this contributes to androgen-dependent cell proliferation or survival has not been determined.

Paradigm 4: Androgen/AR and AR-coregulators regulate the expression and activity of growth factors and growth factor receptors. Androgen increases expression of GFRs such as EGFR or growth factors such as KGF, IGF, EGF, TGFα, or VEGF through enhancing the stability of mRNA expression or through increases in gene transcription via AR co-activators [Wu et al., 2005b]. Thus, GFRs could be involved in an autocrine loop to perpetuate the activity of AR. In addition, it is plausible that androgens, like estrogens or glucocorticoids, regulate the stability of integrins [Ing, 2005].

Androgens not only stimulate the release of paracrine stromal factors, but may also regulate their activation. We demonstrated that androgen suppression causes decreased expression of hepatocyte activator inhibitor (HAI-1) in basal and intermediate cells of normal prostate epithelium [Knudsen et al., 2005]. HAI-1, a transmembrane serine protease inhibitor, is activated by androgen-stimulated cleavage from the cell surface [Martin et al., 2004]. It is an inhibitor of Matriptase and Hepsin, which is over expressed in human prostate cancer metastasis and drives prostate metastasis to mouse bone [Dhanasekaran et al., 2001; Oberst et al., 2001; Klezovitch et al., 2004; Herter et al., 2005]. HGF, the ligand for the c-Met receptor, is activated through proteolytic cleavage by Matriptase/Urokinase and as discovered recently, also by Hepsin [Herter et al., 2005; Kirchhofer et al., 2005]. Thus, HGF activity is regulated by androgen through HAI-1. Thus, through regulating the activation and localization of HAI-1, androgen indirectly modulates the activity of the HGF/c-Met axis.

THERAPEUTIC OPPORTUNITIES BASED ON ANDROGEN-REGULATED GROWTH FACTOR EXPRESSION AND CELL ADHESION IN PROSTATE CANCER

Combination Therapies With Androgen Ablative Treatment

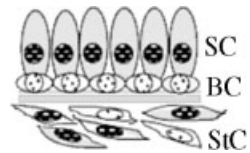
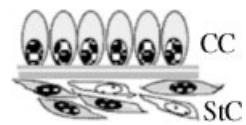
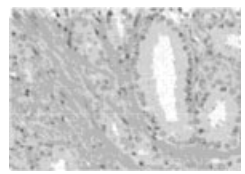
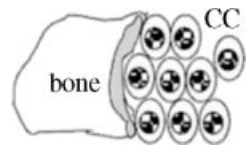
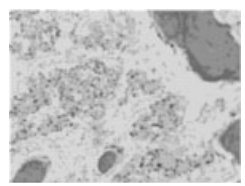
An attractive conceptual approach for treating advanced prostate cancer is to administer androgen-ablative treatment, and to simultaneously target pro-survival proteins that are upregulated as a consequence of androgen deficiency. In addition to Bcl-2, the expression of Clusterin, Hsp 27 and IGFBP-2 and -5 increases in prostate cancer cells upon androgen suppression. Interestingly, these proteins strengthen cell adhesion. The increased cell adhesion may provide a substantial survival impulse and reduce the dependence on androgen for viability. Under conditions of stress, such as during androgen deficiency, chemotherapy, and radiation therapy, cancer cells survive through upregulation of cell adhesion pathways that when targeted lead to their death.

The secreted form of Clusterin is glycosylated, deposited in the extracellular matrix, and affects cell adhesion. The precise mechanism by which extracellular Clusterin mediates cell survival has not been elucidated, but a mechanism for intracellular-expressed Clusterin was discovered recently. In preclinical models of prostate cancer, Clusterin antisense improved the efficacy of chemotherapy, radiation, and androgen withdrawal [Miyake et al., 2000]. Hsp27 localizes to focal adhesions, where it binds the AR coregulator ARA55/Hic-5 [Jia et al., 2001]. Various inhibitors affect subcellular localization and phosphorylation of Hsp27, thereby increasing the network of actin stress fibers and numbers of focal adhesions. Thus, an advantage of the Hsp27 antisense strategy is that it may affect cancer cells directly and also inhibits tumor growth through anti-angiogenic activity [Gleave et al., 2005]. IGFBP-5 and IGFBP-2 are two members of the IGFBP family of proteins whose expression increases upon androgen suppression. IGFBP-2 and -5 expression rose in the Shionogi and LNCaP xenografts, as well as in prostate epithelium in vivo when androgen levels are reduced. Our data demonstrate increased expression of IGFBP-5 in castrated mouse bone and bone marrow, suggesting that IGFBP-5 could act as a paracrine growth factor for metastatic prostate cancer cells (Knudsen, unpublished data). Thus, targeting IGFBP-5 would affect both the cancer and its environment. [Chi and Gleave, 2004].

Integrin Targeted Therapies

A humanized monoclonal antibody with specificity for integrin αvβ3 has been tested in clinical trials [Posey et al., 2001; McNeel et al., 2005]. αvβ3 is expressed on sprouting blood

TABLE I. Substratum Adhesion and Response to Androgen Stimulation of Normal Prostate Tissue and of Prostate Cancer

	A-responsive stromal cells	Type of A-responsive epithelial cells	Substratum adhesion of A-responsive epithelial cell	Epithelial response to androgen stimulation	Schematic representation of responsive epithelium	H&E image
Development	Prostate	Basal/intermediate (AR negative)	Yes	Branching morphogenesis		
Adult	Prostate	Secretory (AR positive)	No	Secretion		
1° Cancer	Prostate	Cancer (AR positive)	Yes	Proliferation, survival, secretion		
Metastasis	Bone	Cancer (AR positive)	Yes	Proliferation, survival, secretion		

, Proliferating cells; , AR expressing cells; , AR positive nuclei; , AR negative nuclei; BC, basal epithelial cells; SC, secretory epithelial cells; StC, stromal cells; CC, cancer cells.

In human tissues, the effects of androgen are appreciated by contrasting histological and immunohistochemical features in tissues from individuals with normal versus suppressed androgen levels. **Basal epithelial cells** are AR negative. They proliferate during development in response to androgenic factors from the stroma and differentiate into secretory cells. In the adult, androgen suppression results in expansion of the basal cell compartment. **Secretory epithelium** expresses highest AR levels. Androgen stimulates terminal differentiation, growth arrest and protein secretion. Androgen deprivation decreases differentiation and secretion and ultimately induced cell death. **Stromal cells** secrete factors in response to androgen that stimulate ductal development. Branching morphogenesis of epithelium during development requires a coordination of proliferation, differentiation and stromal invasion that is stimulated by androgen-regulated stromal factors. Most **primary prostate cancer cells** require androgen for proliferation, differentiation, survival and secretion. Consequently androgen deprivation in patients

vessels and the rationale for this targeted treatment approach is the inhibition of angiogenesis. The concept for using integrin-directed angiogenesis to inhibit the growth of metastatic prostate cancer is supported in an elegant SCID-human-bone model of prostate cancer bone metastasis [Nemeth et al., 1999]. In this animal model, the growth of PC3 cells implanted in fragments of human bone was inhibited by administration of a human-specific anti-αvβ3. The antibody reduced the growth of human-derived blood vessels and the recruitment of osteoclasts by the tumor [Nemeth et al., 2003]. PC3 cells express preferentially αvβ1 and αvβ5 integrins and an αv siRNA caused an increase in tumor cell apoptosis in PC3 mouse bone xenografts [Bisanz et al., 2005]. Once the integrin repertoire of metastatic prostate cancer cells has been fully characterized, there is hope that additional integrin targets suitable for therapeutic development will be identified. The combined inhibition or cytotoxicity of multiple cell types, including the tumor, will be an effective approach in the treatment of metastatic prostate cancer.

SUMMARY

Cell adhesion to the substratum is a critical cofactor for proliferation and survival of epithelial cells. During the development of prostate cancer, malignant luminal epithelial cells transition from cell–cell adhesion to cell–substratum adhesion. In normal epithelium signals from cell adhesion and AR are separated into different cell layers; however, in cancer cells they are co-expressed. Therefore, the engagement of integrins in prostate cancer cells, namely α6β1 and α3β1 and their prostate cancer variants β1A and α6p, may drastically alter the cells' interpretation of growth factor signals and the activity of the AR. Signals from cell surface integrins and GFRs increase during tumor progression and interact with the AR to modulate its transcriptional activity through AR phosphorylation, AR co-activator regulation, or through regulation of other transcription factors, such as FOXO, β-catenin, and NFκB. These interactions may be responsible for changing the functional activity of the AR from differentiation and secretion in normal epithelium to proliferation and survival in cancer. Despite the notion that cell adhesion is a critical component of prostate cancer progression, there is currently little known about the changes in integrin expression during prostate cancer progression and in metastatic cancer cells. Due to the absence of tissue-based analysis, we can only speculate about the role of integrins in mediating tumor growth and progression to androgen-independent, treatment-refractory prostate cancer. Interestingly, recently identified treatment targets that are upregulated by androgen suppression have an impact on cell adhesion. It is likely that integrin expression increases on the surface of metastatic cancer cells and there maybe forms of integrins that are cancer specific; therefore, these cell surface receptors may constitute promising therapeutic targets. Thus, studying their expression and function in locally invasive and metastatic prostate cancer is critical for the development of better therapeutic approaches against metastatic prostate cancer.

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Inhibition of Integrin-mediated Crosstalk with Epidermal Growth Factor Receptor/Erk or Src Signaling Pathways in Autophagic Prostate Epithelial Cells Induces Caspase-independent Death

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In vivo in the prostate gland, basal epithelial cells adhere to laminin 5 (LM5) via $\alpha3\beta1$ and $\alpha6\beta4$ integrins. When placed in culture primary prostate basal epithelial cells secrete and adhere to their own LM5-rich matrix. Adhesion to LM5 is required for cell survival that is dependent on integrin-mediated, ligand-independent activation of the epidermal growth factor receptor (EGFR) and the cytoplasmic tyrosine kinase Src, but not PI-3K. Integrin-mediated adhesion via $\alpha3\beta1$, but not $\alpha6\beta4$ integrin, supports cell survival through EGFR by signaling downstream to Erk. PC3 cells, which do not activate EGFR or Erk on LM5-rich matrices, are not dependent on this pathway for survival. PC3 cells are dependent on PI-3K for survival and undergo caspase-dependent death when PI-3K is inhibited. The death induced by inhibition of EGFR or Src in normal primary prostate cells is not mediated through or dependent on caspase activation, but depends on the induction of reactive oxygen species. In addition the presence of an autophagic pathway, maintained by adhesion to matrix through $\alpha3\beta1$ and $\alpha6\beta4$, prevents the induction of caspases when EGFR or Src is inhibited. Suppression of autophagy is sufficient to induce caspase activation and apoptosis in LM5-adherent primary prostate epithelial cells.

INTRODUCTION

In vivo, the precise regulation of epithelial cell homeostasis involves interactions between cells and their microenvironment. Cells receive signals from both the extracellular matrix in the basement membrane and soluble factors secreted by the stroma that precisely control the timing of cell division, growth arrest, differentiation, and survival. Integrins on the cell surface that interact with laminin 5 (LM5) in the extracellular matrix, such as $\alpha3\beta1$ and $\alpha6\beta4$, are critically involved in mediating survival. Genetic loss of LM5, or its receptors $\alpha3$, $\alpha6$, or $\beta4$ integrins, in vivo results in cell detachment and induction of caspase-mediated apoptosis, even in the presence of soluble factors (Ryan *et al.*, 1999; DiPersio *et al.*, 2000). This detachment-induced form of apoptosis has been termed anoikis (Frisch and Screaton, 2001). In vitro anoikis can be rescued by expression of an activated form of FAK, Rac, or Akt (Frisch *et al.*, 1996; Rytomaa *et al.*, 2000; Coniglio *et al.*, 2001), suggesting that integrin-mediated signaling through these molecules is required to maintain cell survival. However, studies in which specific signaling pathways are inhibited while integrins are still engaged suggest alternative pathways, such as Ras/Erk or Jnk, are required for integrin-mediated survival (Almeida *et al.*, 2000; Manohar *et al.*, 2004). Whether signaling from multiple pathways is involved in mediating integrin-dependent survival and whether different pathways are unique to specific cell types have not been extensively investigated.

In addition to classical caspase-mediated apoptosis, such as that observed during anoikis, several other mechanisms of cell death have been described (Melino *et al.*, 2005). Other forms of cell death include caspase-independent cell death, autophagy, or cornification. The role of integrins in regulating cell survival through suppression of these other death pathways is unknown. However, some of the same integrin-induced signal transduction pathways that have been linked to survival are also important for regulating these alternative cell death pathways. For example the Ras/Erk and PI-3K pathways act as positive and negative regulators, respectively, of autophagy in several cell types (Kondo *et al.*, 2005). Additionally, epithelial cells have been shown to undergo death by cornification in response to inhibition of Erk and Jnk, but not PI-3K (Uzgare and Isaacs, 2004). Finally, death induced by over expression of Ras, or suppression of Raf in melanoma cells leads to caspase-independent cell death (Chi *et al.*, 1999; Panka *et al.*, 2006). Whether integrin-induced activation of specific signaling pathways plays a role in regulating any of these cell death mechanisms has not been determined.

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Abbreviations used: EGFR, epidermal growth factor receptor; PEC, prostate epithelial cells; LM, laminin; PI-3K, phosphatidyl inositol 3-kinase.

Although studies with various established cell lines have been extremely useful for elucidating potential signaling pathways involved in integrin-mediated survival, it is important to place the findings in the context of a defined organ system where the specific cell type, the integrins expressed, and the matrix being studied are better defined. Basal epithelial cells in the prostate gland express $\alpha6\beta4$ and $\alpha3\beta1$ integrins and adhere to a basement membrane rich in LM5 (Knox *et al.*, 1994). When these cells are placed in culture they retain in vitro a majority of the properties seen in vivo, including the ability to secrete and organize their own LM5-rich matrix (Gmyrek *et al.*, 2001; Yu *et al.*, 2004).

Our work and that of others have demonstrated that integrin engagement is sufficient to activate receptor tyrosine kinases (Plopper *et al.*, 1995; Miyamoto *et al.*, 1996; Wang *et al.*, 1996; Moro *et al.*, 1998; Danilkovitch-Miagkova *et al.*, 2000; Kuwada and Li, 2000; Marcoux and Vuori, 2003; Bill *et al.*, 2004). We demonstrated that adhesion of normal epithelial cells to matrix is sufficient to induce activation of the epidermal growth factor receptor (EGFR), independently of ligand (Bill *et al.*, 2004). In addition, we demonstrated that integrin-mediated activation of a subset of signaling pathways, namely the Ras/Erk and PI-3K/Akt pathways, are dependent on integrin-induced EGFR activation. Because both of these pathways have been implicated in regulating integrin-mediated survival, we hypothesized that integrin-mediated survival of epithelial cells via Ras/Erk or PI-3K/Akt pathways could be mediated through integrin-dependent activation of EGFR. To test this hypothesis, we assessed the ability of primary prostate epithelial cells (PECs) adherent to their endogenous LM5-rich matrix to survive in the context of EGFR and downstream signaling inhibitors.

MATERIALS AND METHODS

Antibodies

EGFR immunoprecipitating and blocking monoclonal antibodies were purified in the Monoclonal Antibody Core at VARI from hybridoma cells obtained from American Type Culture Collection (ATCC; Manassas, VA; HB-8508). EGFR (Ab12) immunoblotting antibodies were purchased from NeoMarkers (Fremont, CA). Erk and p130Cas antibodies were purchased from Becton-Dickinson Transduction Labs (Lincoln Park, NJ). Phospho-specific antibodies against Erk1/2 (T202/Y204) and Akt (S473) and antibodies to Bcl-2 and Bcl-XL were purchased from Cell Signaling (Beverly, MA). The anti-phosphotyrosine mAb 4G10 was obtained from Upstate Biotechnology (Lake Placid, NY). The Akt antibody was described previously (Bill *et al.*, 2004). Blocking antibodies for $\beta4$ integrin (ASC-8) and $\alpha3$ integrin (P1B5) were purchased from Chemicon (Temecula, CA) and GoH3 $\alpha6$ integrin antibody was obtained from Becton-Dickinson.

Cell Culture

Primary cultures of human PECs were derived from normal human prostatic tissue and cultured as described previously (Gmyrek *et al.*, 2001). Human samples were obtained after institutional IRB approval. PECs were maintained in Keratinocyte-SFM medium (Invitrogen, Carlsbad, CA) supplemented with bovine pituitary extract and epidermal growth factor (EGF). All experiments were conducted on cells between passages 3 and 5. PC3 cells were obtained from ATCC. PC3 cells were maintained in F12K medium (Invitrogen) supplemented with 10% fetal bovine serum, 2 mM glutamine, 50 U of penicillin, and 50 mg of streptomycin/ml.

Integrin Signaling

Preparation of cells for adhesion to extracellular matrices was carried out as described in Miranti (2002). Briefly, cells were growth factor-starved for 48 h, trypsinized, treated with soybean trypsin inhibitor (Invitrogen), washed in PBS, and placed in suspension in growth factor-free medium for 30–60 min. Cells were then either plated on tissue culture plates blocked with 1% BSA (Sigma, St. Louis, MO) to allow deposition of endogenous LM5-rich matrix or directly replated on LM5-coated plates obtained from culturing PECs as described previously for LM5-secreting cells (Xia *et al.*, 1996). In some cases, PC3 cells were also plated on laminin 1 (LM1; Invitrogen). Similar results were obtained in PC3 cells on LM1 as on LM5. Occasionally cells were also treated with 2–10 ng/ml EGF (Upstate Biotechnology) or 50 ng/ml HGF (Calbiochem, La Jolla, CA) for 10 min. A suspension control was maintained at 37°C. Two hours after plating on the matrix cells were lysed either in Triton X-100 (50 mM Tris, pH 7.5, 100 mM NaCl, 0.5 mM EDTA, 1% Triton X-100, 50 mM NaF, 50 mM β-glycerophosphate, 5 mM sodium pyrophosphate, 1 mM Na_3VO_4, 1 mM PMSF, 100 U/ml aprotinin, 10 μg/ml pepstatin, and 10 μg/ml leupeptin) or RIPA (10 mM Tris, pH 7.2, 158 mM NaCl, 1 mM EDTA, 0.1% SDS, 1% NaDOC, 1% Triton X-100, 1 mMNa_3VO_4, 1 mM PMSF, 100 U ml aprotinin, 10 μg/ml pepstatin, and 10 μg/ml leupeptin) buffers. Pharmacological inhibitors, PD168393, AG1478, LY294002, SU6656, or PP2, purchased from Calbiochem, were added to suspension cells 20 min before plating on matrix; except for SU6656, which was added 16 h before placing cells in suspension. All working concentrations of the pharmacological inhibitors were determined by titrating to the minimum inhibitor concentration that effectively blocked the target of the pharmacological inhibitor for the duration of our experiments. Inhibitor effectiveness was monitored by Western blotting. Specifically, PD168393 and AG1478 were tested for their ability to inhibit EGFR tyrosine phosphorylation, p130Cas tyrosine phosphorylation (a Src substrate) was used to test SU6656 and PP2, phosphorylation of Akt was used for LY294002, and U0126 was tested against phosphorylated Erk. Titrations were performed for each drug in each cell type.

Antibody Blocking Assays

Blocking Integrins. For integrin blocking studies, PECs were starved and placed in suspension and then plated on 1% bovine serum albumin (BSA)-blocked eight-chamber slides in the presence of 10 μg/ml blocking anti-$\beta4$ integrin antibody (ASC-8), anti-$\alpha3$ integrin antibody (P1B5), anti-$\alpha6$ integrin antibody (GoH3), or IgG. Cells were allowed to adhere to endogenous LM5-rich matrix for 48–72 h in the presence of the indicated antibodies. Cells were monitored for viability by Annexin V staining, caspase activation, autophagy induction with LC3-GFP, or for Erk activation by immunoblotting.

Blocking EGF Binding. Thirty minutes before plating, growth-factor–starved PEC suspension cells were pretreated with 0–10 μg/ml mAb to EGFR (AB225 [HB-8508, ATCC]) or 10 μg/ml nonspecific mouse IgG for 30 min with occasional mixing. Cells were allowed to adhere to LM5-rich matrix in the presence or absence of 2 ng/ml EGF for 2 h. Cells were lysed, and EGFR tyrosine phosphorylation and Erk activation were monitored in immunoprecipitates or cell lysates, respectively.

Cell Survival Assays. PECs were starved and placed in suspension as described above and then plated on 1% BSA-blocked tissue culture plates to allow deposition and adhesion to endogenous LM5 matrix. Serum-starved PC3 cells were plated on 1% BSA-blocked tissue culture plates precoated with 10 μg/ml laminin. Pharmacological inhibitors, 1 μM staurosporine (Promega, Madison, WI), 0.5 μM PD168393, 1 μM AG1478, 10 μM U0126, 10 μM LY294002, 0.5–2 μM SU6656, 10 μM PP2, 50 μM butylated hydroxyanisole (BHA), 1.25 mM *N*-acetylcysteine (NAC), or 10 mM 3-methyladenine (3MA), were then added. Cells were allowed to adhere for 4 h and then nonadherent cells were removed and drugs were replaced. Cells were incubated for an additional 72 h. LY294002 was replenished 48 h after plating.

To assess cell death, cells were stained with Annexin V using a kit obtained from Molecular Probes (Invitrogen). Staining was carried out according the supplied protocols. For all staining procedures both attached and floating cells were collected. Attached cells were removed by trypsinization and pooled with floating cells, and all cells were washed one time. For Annexin V staining, cells were resuspended in Annexin binding buffer (10 mM HEPES, 140 mM NaCl, 2.5 mM CaCl, pH 7.4) containing Alexa-fluor–conjugated Annexin V and incubated in the dark for 15 min. Samples were put on ice and immediately analyzed. Extent of staining was monitored by fluorescence-activated cell sorting (FACS) using a FACS Caliber (Becton-Dickinson) and CellQuest version 3.1.3 acquisition and analysis software (Becton-Dickinson) immediately after staining. On several occasions Annexin V staining was also monitored in adherent cells (without trypsinization) by microscopy using a Nikon Eclipse TE300 fluorescence microscope (Melville, NY) and OpenLab version 3.1.7 image analysis software (Improvision, Lexington, MA).

Caspase Activity Assays. Caspase 3 and 7 activity in PEC and PC3 cells was directly measured using a CaspaseGlo 3/7 kit (Promega) following the manufacturer's suggested protocol. For PECs 10,000 cells/well were plated on endogenous LM5 in BSA-coated 96-well plates in the presence of DMSO, 1 μM staurosporine (Promega), 0.5 μM PD168393, 10 μM PP2, or 10 mM 3MA, with or without 20 μM zVAD (Promega). For PC3 cells, 10,000 cells/well were plated on 1% BSA blocked 96-well plates precoated with 10 μg/ml laminin, respectively. Cells were plated in the presence of DMSO, 1 μM staurosporine, 0.5 μM PD168393, 10 μM LY294002, 2 μM SU6656, and 10 μM PP2. CaspaseGlo reagent was added at various times after inhibitor treatment and incubated for 1 h at room temperature in the dark. Relative light intensity was measured in each well using a Fluoroskan Assent FL fluorometer and software (Labsystems, Franklin, MA).

Immunoprecipitation and Immunoblotting. Immunoprecipitation mixtures containing 500–1000 μg protein were incubated with the appropriate anti-

bodies for 3 h at 4°C with either protein A– or protein G–conjugated agarose beads (Pierce, Rockford, IL) to capture the complexes. All immunoprecipitated complexes were washed three times with their respective lysis buffer. Immunoprecipitated samples from adhesion assays were resuspended in 2× SDS sample buffer. In some cases 50–75 μg of total cell lysates were placed directly in 2× SDS sample buffer. All resuspended samples were boiled and subjected to SDS-PAGE, transferred to a polyvinylidene difluoride (PVDF) membrane. The PVDF membranes were blocked with 5% BSA in Tris-buffered saline containing 0.1% Tween 20 (TBST) for 2 h, followed by a 2-h incubation with the appropriate primary antibodies in 5% BSA/TBST. After several washes, blots were incubated with a horseradish peroxidase–conjugated secondary antibody (Bio-Rad, Richmond, CA) for 1 h in 5% BSA/TBST and visualized with a chemiluminescence reagent and captured by a CCD camera in a Bio-Rad Chemi-Doc Imaging System. Levels of activation, relative to total levels of protein, from blots captured by CCD camera were quantified using Quantity One software (Bio-Rad). Blots were stripped in low-pH 2% SDS at 65°C for 60 min, rinsed, and reprobed for total levels of protein in the immunoprecipitates or cell lysates.

Autophagy Assay. LC3-GFP in the pBABE expression vector was kindly provided by Dr. Jay Debnath (University of California, San Francisco, CA). The LC3-GFP cDNA BamHI/SalI restriction fragment was subcloned into the BglII and SalI restriction sites of pShuttle-CMV (Strategene, La Jolla, CA). pAd-Easy (Strategene) adenoviral recombinants containing LC3-green fluorescent protein (GFP) were generated in BJ5183-AD1 bacteria. HEK293 cells were transfected with adenoviral recombinant DNA and adenoviruses purified using a kit from Clontech and titrated by GFP expression in PECs.

PECs were infected at an moi of 2 with adenoviruses expressing LC3-GFP fusion protein. Twenty-four hours later, cells were growth factor-starved or left in complete medium and allowed to adhere to their own LM5. For antibody blocking experiments, antibodies were added at the time plating on matrix. Localization of LC3-GFP was monitored by standard fluorescence microscopy at 24 and 48 h after plating using a Nikon Eclipse TE300 fluorescence microscope and OpenLab version 3.1.7 image analysis software (Improvision).

RESULTS

Integrin Engagement in PECs Activates EGFR Independent of Ligand

Primary PECs derived from prostectomy tissues, when placed in serum-free culture, secrete and organize an extracellular matrix containing LM5 within 2–3 h after plating (Yu *et al.*, 2004). Adhesion of PECs to this LM5-rich matrix induces activation of the ErbB family receptor tyrosine kinase EGFR (Figure 1A) and its downstream target Erk (Figure 1B). Adhesion to the LM5-rich matrix failed to stimulate detectable levels of Akt activation (Figure 1C). However, Akt was activated by treatment with EGF or human growth factor (HGF), demonstrating that the PI-3K/Akt pathway is intact in these cells. Inhibition of EGFR activity with the EGFR-specific inhibitors AG1478 (not shown) or PD168393 blocked integrin-induced EGFR tyrosine phosphorylation (Figure 1A) and downstream signaling to Erk (Figure 1B). Thus integrin-mediated activation of Erk on LM5-rich matrix is dependent on EGFR.

Src kinases have been implicated in regulating integrin-mediated activation of receptor tyrosine kinases (Danilkovitch-Miagkova *et al.*, 2000; Moro *et al.*, 2002); however, in PECs, inhibition of Src activation by the Src kinase-specific inhibitor SU6656 (Blake *et al.*, 2000) had no effect on EGFR activation or its downstream target Erk (Figure 1, A and B). Inhibition of Src did block integrin-mediated activation of its downstream substrate p130Cas (Figure 1D). Reciprocally inhibition of EGFR did not block integrin-mediated tyrosine phosphorylation of the Src substrate p130Cas. These data demonstrate that adhesion of PECs to LM5-rich matrix regulates two independent signaling pathways: one that activates EGFR, independent of ligand, and signals to Erk and one that activates Src and its downstream target p130Cas, independently of EGFR.

To rule out the possibility that residual EGF was responsible for activation of EGFR and Erk, we used a blocking antibody that prevents EGF binding to EGFR, and thus EGFR activation by EGF. Stimulation of PECs with EGF

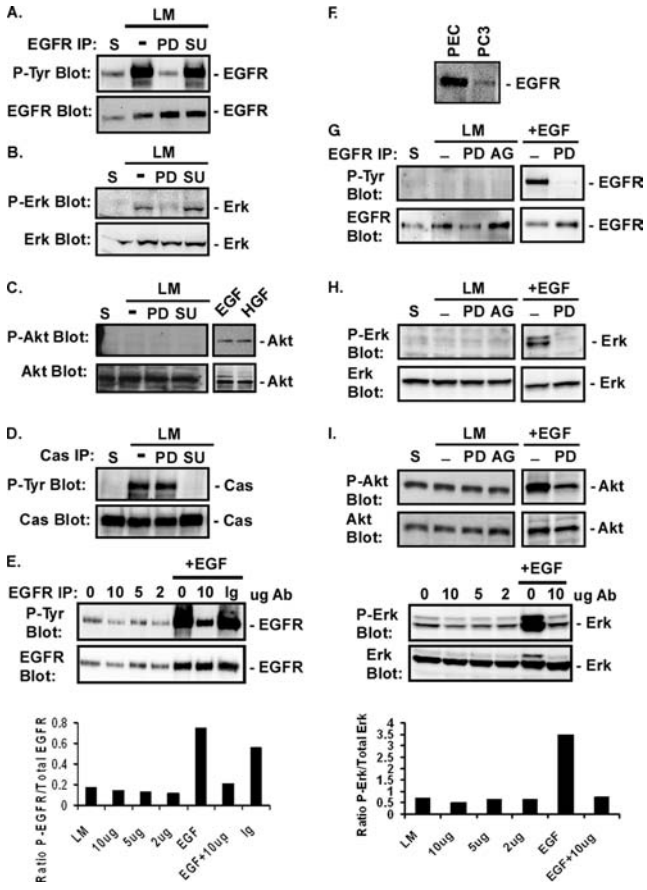

Figure 1. Integrin-mediated signaling on LM5 in PECs. (A–E) Growth factor-starved PECs were placed in suspension (S) and treated with DMSO (−), 0.5 μM PD168393 (PD), or 0.5 μM SU6656 (SU) before plating on LM5 (LM) for 2 h. (A) EGFR or (D) p130Cas were immunoprecipitated (IP) and levels of tyrosine phosphorylation were monitored by immunoblotting with anti-phosphotyrosine antibodies (P-Tyr Blot). (B) Erk and (C) Akt activation were monitored by immunoblotting of whole cell extracts with the anti-Erk (T202/Y204) and anti-Akt (S473) phospho-specific antibodies (P-Erk Blot, P-Akt Blot). Some cells were treated with 10 ng/ml EGF or 50 ng/ml HGF for 10 min. (E) PECs placed in suspension were left untreated (0), treated with 2–10 μg anti-EGFR antibody (μg Ab), or treated with nonspecific IgG (Ig) for 30 min before plating on LM5. At the time of plating 2 ng/ml EGF was added (+EGF) to some samples. EGFR activation was monitored in immunoprecipitates by immunoblotting with anti-phosphotyrosine antibodies (P-Tyr Blot). Erk activation was monitored by immunoblotting of whole cell extracts with anti-Erk phospho-specific antibodies (P-Erk Blot). Total levels of each protein in the immunoprecipitates and cell lysates were measured by immunoblotting of stripped blots with the indicated antibodies. Levels of EGFR and Erk activation relative to total protein levels were quantified using Quantity One software (Bio-Rad) and are displayed to the right of the respective blots. (F) Fifty micrograms of whole cell extracts from PEC or PC3 cells were analyzed for EGFR levels by immunoblotting. (G–I) PC3 cells were serum-starved and placed in suspension and treated with DMSO (−), 0.5 μM PD168393, or 1 μM AG1478 before plating on LM5 (LM) for 1 h. Some cells were also treated with 2 ng/ml EGF (+EGF) for 10 min. (G) EGFR was immunoprecipitated (IP) and levels of tyrosine phosphorylation monitored by immunoblotting (P-Tyr Blot). (H) Erk and (I) Akt activation were monitored by immunoblotting of whole cell extracts with anti-Erk and anti-Akt phospho-specific antibodies (P-Erk Blot, P-Akt Blot).

effectively activates EGFR and Erk in PECs. Pretreatment of PECs with the EGFR blocking antibody did not significantly inhibit integrin-induced activation of EGFR or Erk (Figure 1E). In contrast, the same EGFR-blocking antibody blocked EGF-induced activation of EGFR and Erk, thereby reducing its activation to similar levels seen on matrix alone (Figure 1E). These data indicate that the ability of integrins to activate EGFR occurs independently of EGFR ligand.

The ability of integrins to activate Erk in epithelial cells is dependent on EGFR activation and is regulated in part by the level of EGFR expression (Moro *et al.*, 1998; Bill *et al.*, 2004). For instance, over expression of EGFR in fibroblasts (where Erk activation is not dependent on EGFR) leads to EGFR-dependent Erk activation in fibroblasts (Moro *et al.*, 1998). Adhesion of the epithelial cell line PC3, which expresses threefold less EGFR than PECs (Figure 1F), to the LM5-rich matrix produced by PECs fails to activate EGFR or Erk (Figure 1, G and H). EGFR is active in PC3 cells because stimulation with EGF activates both EGFR and Erk. In contrast, adhesion to matrix does not increase Akt activation, which is partially constitutively activated (Figure 1I). This is likely due to the loss of Pten expression in these cells (Vlietstra *et al.*, 1998). Consequently, constitutively active Akt is not blocked by EGFR inhibition. Thus, we predict that survival of these two cell lines on LM5-rich matrix is likely to be mediated by different signaling pathways.

EGFR and Src Independently Regulate Integrin-mediated Cell Survival in PECs

Treatment of LM5-rich adherent PECs with two EGFR-specific inhibitors, AG1478 or PD168393, results in the induction of cell death as measured by Annexin V staining (Figure 2, A and B). Maximal Annexin V staining is observed 72 h after drug treatment and occurs in over 85% of the cells (Figure 2A). Inhibition of Erk activation, the downstream target of EGFR, with U0126, but not inhibition of PI-3K with LY294002, induced cell death to the same extent as loss of EGFR signaling (Figure 2C). Together with the signaling data shown in Figure 1 these findings indicate that Erk signaling downstream of EGFR is required for PEC survival on LM5-rich matrix. Inhibition of Src by SU6656 or PP2 also induced cell death (Figure 2, B and C), with a time course and effectiveness that is similar to that seen with EGFR or Erk inhibition. Simultaneous inhibition of EGFR and Src did not increase the amount of cell death observed (Figure 2C), suggesting that although these molecules lie on separate signaling pathways (see Figure 1), they may regulate cell survival through a similar downstream mechanism. All drugs were effective at inhibiting their respective signaling pathways at the concentrations used (see *Methods and Materials*).

Integrin-mediated Survival in PC3 Cells Is Not EGFR-dependent

In our studies we observed that adhesion of PC3 cells to LM5 (or LM1), does not induce the activation of EGFR or signal downstream to activate Erk (see Figure 1, G and H). Accordingly, inhibition of EGFR does not induce significant cell death in PC3 cells as measured by Annexin V staining (Figure 2D).

PC3 cells do not express the PI-3K/Akt inhibitor Pten, and consequently Akt is activated independent of matrix in PC3 cells and is not blocked when EGFR signaling is inhibited (Figure 1H). However, inhibition of the PI-3K pathway with LY294002 induced cell death in LM-adherent PC3 cells (Figure 2D). The extent of cell death induced by inhibition of PI-3K in PC3 cells was only twofold compared with the fourfold increase observed with inhibition of EGFR in PECs, suggesting that other mechanisms might be involved in regulating PC3 cell survival on matrix. We blocked Src signaling in PC3 cells with either SU6656 or PP2 and found that integrin-mediated survival in PC3 cells, like PECs, is also dependent on Src (Figure 2D). Inhibition of Src resulted in a three to fourfold increase in cell death. Thus cells that are able to activate EGFR/Erk signaling on LM5 are dependent on this pathway for survival. Cells unable to activate the

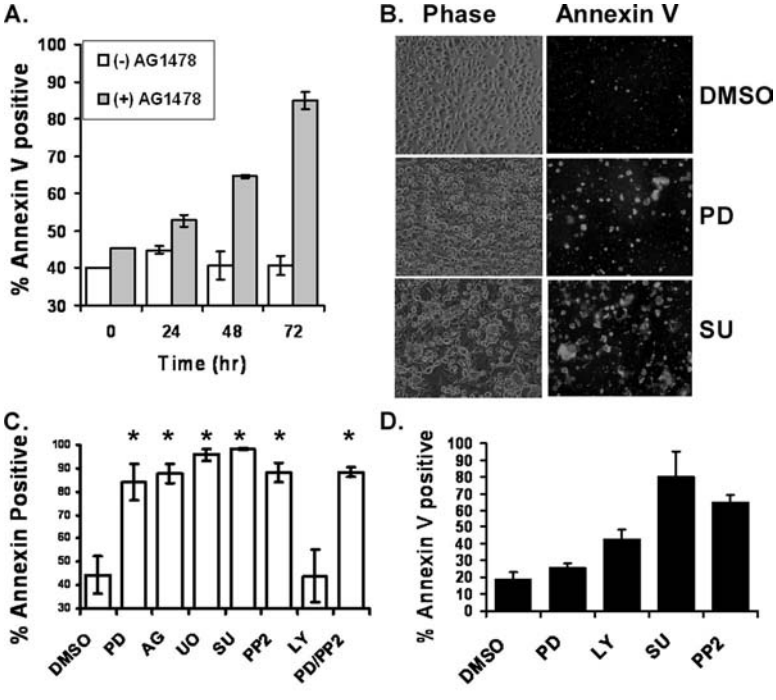

Figure 2. Integrin-induced activation of EGFR and Src is required for LM5-mediated survival. PECs were growth factor-starved for 48 h and placed in suspension for 30 min. Cells were allowed to adhere to LM5 in the absence or presence of 1 μM AG1478 (AG), 0.5 μM PD168393 (PD), 10 μM U0126 (U0), 10 μM LY294002 (LY), 0.5 μM SU6656 (SU), or 10 μM PP2 (PP2) for 4 h and then nonadherent cells were washed away, and drugs were replaced. (A) Percent Annexin V staining was quantified by FACS at 0, 24, 48, or 72 h after inhibition of EGFR with AG1478 (AG) or (B) visualized by phase-contrast and fluorescence microscopy after 72 h of treatment with PD168393 (PD) or SU6656 (SU). (C) Percent Annexin V staining 72 h after treatment of PECs with PD168393 (PD), AG1478 (AG), U0126 (U0), SU6656 (SU), PP2 (PP2), LY924002 (LY), or both PD and PP2 was quantified by FACS. (D) Growth factor-starved PC3 cells were placed in suspension and pretreated with DMSO, 0.5 μM PD168393 (PD), 10 μM LY294002 (LY), 2 μM SU6656 (SU), or 10 μM PP2 (PP2). Cells were analyzed for percent Annexin V staining by FACS 72 h after plating on laminin. Error bars, SD; n = 4; *$p < 0.001$ compared with DMSO control.

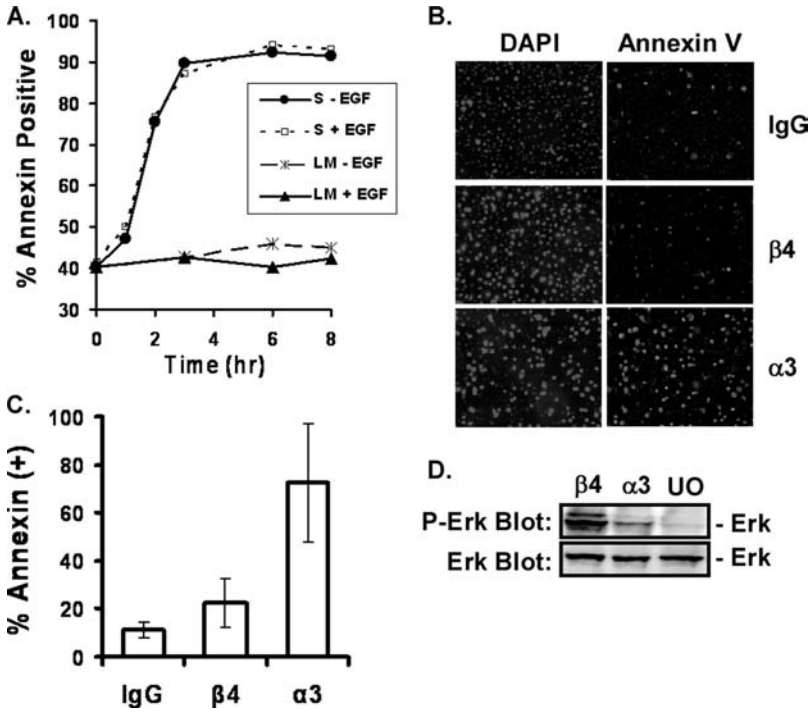

Figure 3. α3 integrin regulates survival in PECs. (A) PECs were kept in suspension in complete medium for 8 h. At 0, 1, 2, 3, 6, and 8 h after being suspended, a sample of cells was removed and analyzed for Annexin V positivity by FACS. (B–D) PECs were growth factor-starved for 48 h and placed in suspension for 30 min. At the time of plating on LM5, PECs were treated with anti-integrin antibodies to β4 (β4), α3 (α3), or IgG (IgG). (B) Annexin V staining was monitored 72 h after plating and visualized by fluorescence microscopy. DAPI was used to stain the nuclei (DAPI). (C) Quantification of percent Annexin V staining from B. (D) Cells were pretreated with anti-integrin antibodies (β4, α3), or U0126 (U0) before adhesion to LM5. The levels of Erk activation were measured by immunoblotting of cell extracts with anti-phospho Erk antibodies (P-Erk Blot). Total levels of Erk were monitored by immunoblotting with anti-Erk antibodies (Erk Blot). Error bars, SD.

EGFR/Erk pathway use other signaling pathways, such as PI-3K, for survival.

LM5-dependent Survival through EGFR Is Mediated by Engagement of α3 Integrin in PECs

PECs express α3β1 and α6β4, both of which mediate adhesion to LM5 (Delwel *et al.*, 1994; Niessen *et al.*, 1994). As expected, placing PECs in suspension rapidly induces cell death, with 80–90% of cells displaying Annexin V positivity within 6 h (Figure 3A). To determine which of these integrin receptors, α3β1 or α6β4, is mediating survival, we used specific blocking antibodies raised against α3 or β4 integrin. PECs treated with either α3 or β4 blocking antibodies were still able to adhere to LM5-rich matrix; however, treatment with anti-α3 antibody blocked cell spreading (not shown). Blocking antibody to α3 integrin induced cell death to a similar extent as that seen with inhibition of EGFR or Src (Figure 3, B and C), i.e., 70–80%. Blocking β4 integrin on the other hand did not compromise survival. Furthermore, cells treated with blocking antibodies to α3 integrin, but not β4, were also defective in activating Erk (Figure 3D) and EGFR (not shown). These data indicate that signaling through α3 integrin regulates LM5-mediated activation of EGFR and its subsequent activation of Erk. Thus signaling from LM5 through α3β1 to EGFR and downstream to Erk is critical for regulating survival.

Cell Death in PECs Is Not Caspase-dependent

Loss of adhesion has been associated with a specialized form of caspase-dependent apoptosis known as anoikis. Despite the fact that 85–90% of our cells were Annexin V positive, only 30% were positive by TUNEL and only 14% displayed sub-G0 content based on PI staining (not shown). This suggested that cell death due to loss of integrin signaling, as opposed to loss of adhesion, may proceed via a different mechanism. To determine if PECs were dying via caspase-dependent apoptosis, we first monitored caspase 3 cleavage and loss of Bcl-XL or Bcl-2. Neither loss of Bcl-XL or Bcl-2, nor activation of caspase 3 was detectable by immunoblotting (Figure 4A). We then used an enzyme assay to directly measure caspase activity in dying PECs. Interestingly, in PECs loss of integrin-mediated signaling through EGFR, Src, or both simultaneously, failed to induce significant caspase 3/7 activity throughout a 72-h time course (Figure 4, B and C). Staurosporine is an efficient activator of classical apoptosis and caspases. To demonstrate that PECs were capable of activating caspases, we measured capsase 3/7 activity in staurosporine-treated PECs. A 16-fold increase in caspase 3/7 activity was observed in staurosporine-treated PECs (Figure 4B) as well as a loss of full-length caspase 3 as measured by immunoblotting (Figure 4A). This activity was inhibited by the caspase inhibitor zVAD. Furthermore, inhibiting caspase activity with zVAD was not sufficient to rescue cells from death upon EGFR or Src inhibition (Figure 4, D and E). One possibility is that the Annexin V positive cells are not dying. However, counting the number of remaining live cells after 72 h, as determined by trypan blue exclusion, indicated that 90% of the remaining cells were indeed dead (Figure 4E).

In contrast, induction of apoptosis in PC3 cells by treatment with LY294002 to inhibit PI-3K activity, or SU6656 or PP2 to inhibit Src activity, induced a 2.5-fold increase in caspase activity (Figure 4F).

Cell Death in PECs Is Not Due To Autophagy

Nutrient and serum deprivation can induce cells to enter a state of survival termed autophagy (Lum *et al.*, 2005). Knowing that our experiments were conducted under growth factor starvation conditions, we suspected that the cell death we were observing could be autophagic in nature (Baehrecke, 2005). To address this possibility, we first determined whether placing PECs under starvation conditions was sufficient to induce autophagy. LC3 protein is generally present throughout the cell, and upon induction of autophagy it is processed and incorporated into autophagic vacuoles. PECs were infected with an adenovirus that expresses an LC3-GFP fusion

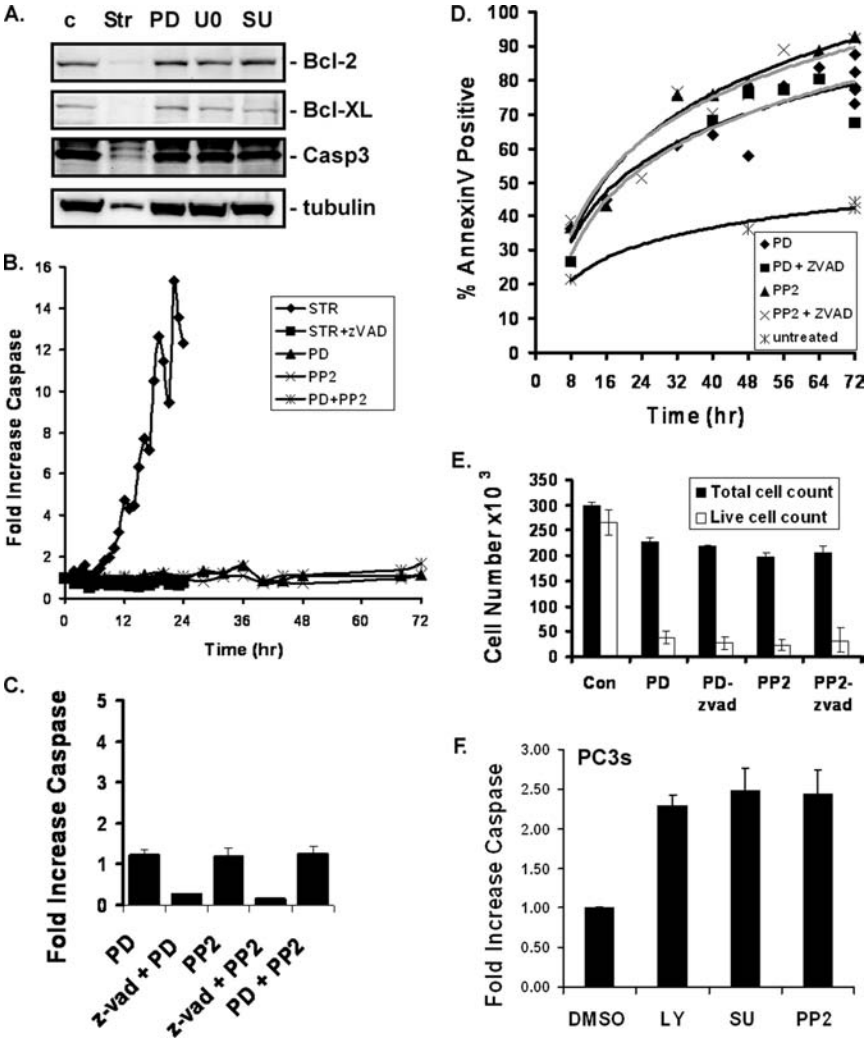

Figure 4. Cell death induced in PECs is caspase-independent. (A) PECs were pretreated with DMSO (c), Staurosporine (Str), PD168393 (PD), U0126 (U0), or SU6656 (SU) and allowed to adhere to laminin for 72 h. The levels of Bcl-XL, Bcl-2, and caspase 3 were monitored by immunoblotting of cell lysates. Total levels of protein in the lysates were monitored by immunoblotting with anti-tubulin. (B) PECs were plated on LM5 and treated with DMSO, 1 μM staurosporine (STR) for 24 h or 0.5 μM PD168393 (PD), 10 μM PP2 (PP2), or both (PD+PP2) for 72 h. The caspase inhibitor z-VAD at 20 μM was added at the time of plating as indicated (zVAD). Caspase 3/7 activity was monitored every 1–4 h. Caspase data are expressed as fold increase in caspase activity over that observed in untreated cells. (C) PECs were drug treated as in B. Caspase 3/7 activity was monitored 72 h after drug treatment. (D) PECs were plated on LM5 and treated with DMSO (untreated), 0.5 μM PD168393 (PD), or 10 μM PP2 (PP2). z-VAD at 20 μM was added at the time of plating as indicated (zVAD). Treated cells were sampled every 4–12 h over a 72-h time course and analyzed for percent Annexin V staining by FACS. (E) PECs were treated as in D. Cells were counted in the absence (total cell count) or presence (live cell count) of trypan blue to determine the total number of live versus dead cells. (F) PC3 cells were plated on laminin, treated with DMSO, 10 μM LY294002 (LY), 2 μM SU6656 (SU), or 10 μM PP2 (PP2). Thirty-six hours later caspase 3/7 activity was measured. Error bars, SDs.

protein. Induction of autophagy is indicated by a shift from very diffuse LC3-GFP fluorescence throughout the cell to punctate fluorescence within the cytoplasm (Boya *et al.*, 2005). As early as 24 h after plating growth factor-starved LC3-GFP expressing PECs on LM5, punctate fluorescence was evident. By 48 h, multiple punctate fluorescent areas were observed in over 90% of cells under growth factor starvation conditions (Figure 5, A and B). Punctate fluorescence was rarely observed (in <10% in cells) in normal growth media at 24 or 48 h after plating on LM5 (Figure 5, A and B). Thus adhesion to LM5-rich matrix in growth factor-deprived cells leads to induction of autophagy. Furthermore, adhesion of growth factor-deprived PECs to their LM5-rich matrix is sufficient to mediate cell survival for at least 8 d. However, removal from matrix and placement in suspension results in maximum Annexin V positivity within 6 h (Figure 3A). Pretreatment of GFP-LC3 expressing cells with integrin-blocking antibodies to α3 integrin resulted in a threefold reduction in LC3 punctate staining versus a 1.5-fold reduction with β4 or α6 blocking antibodies (Figure 5C). Thus adhesion of growth-factor deprived PECs to LM5-rich matrix primarily via $\alpha3\beta1$, but also to some extent through $\alpha6\beta4$, is required to maintain autophagy.

The autophagy inhibitor 3MA is a type-III PI3K-inhibitor that blocks the formation of autophagic vacuoles. We expected that if cell death was due to autophagy, treatment with 3MA would rescue the cells from death. However, inhibiting autophagy in PECs by treatment with 3MA, in the presence of EGFR or Src inhibitors, does not rescue cells from death (Figure 6A). In fact, treatment of starved PECs with 3MA is sufficient to induce cell death. Cell death under autophagic inhibitory conditions is accompanied by caspase activation (Figure 6B). These data indicate that autophagy is acting as an integrin-mediated survival mechanism in LM5-rich matrix adherent PECs. Simultaneous inhibition of autophagy and EGFR/Erk signaling increases caspase activity. Similar results were obtained using bafilomycin (not shown), which inhibits autophagy by inhibiting fusion between autophagosomes and lysosomes by blocking vacuolar H+ ATPase (Yamamoto *et al.*, 1998).

If $\alpha3\beta1$ integrin regulates cell survival by maintenance of autophagy and signaling through EGFR/Erk and if blocking autophagy and EGFR induces caspase-mediated death, then blocking $\alpha3\beta1$ integrin should also induce caspase activation. Cells pretreated with α3 blocking antibodies, but not α6 or β4 blocking antibodies, induced a fivefold increase in caspase 3/7 activity (Figure 6C).

Caspase-independent death has been linked to the generation of reactive oxygen species (ROS) in several cell systems. To determine if the mechanism by which inhibition of EGFR signaling induces cell death is due to the generation of ROS, PECs were pretreated with two different ROS inhibi-

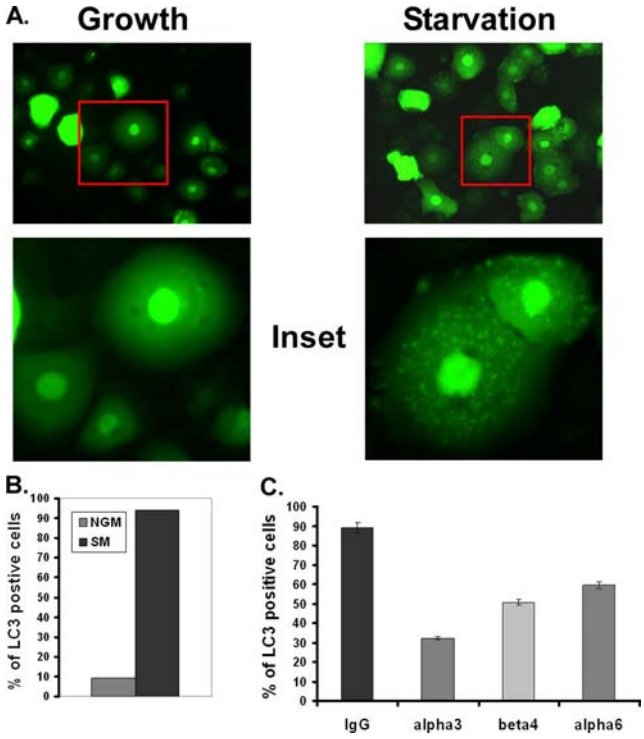

Figure 5. Growth factor starvation of PECs induces autophagy. PECs were infected with an adenovirus to express the LC3-GFP fusion protein, and cells were placed in either normal growth media (growth) or starvation media (starvation). (A) Cells were observed at 20× magnification by fluorescence microscopy to evaluate the pattern of LC3-GFP localization 48 h after plating. Some GFP is seen accumulating in the nucleus under both growth conditions. (B) To rule out the possibility of nonspecific staining artifacts cells displaying at least 10 punctate spots or more in the serum-starved (SM) conditions were scored as positive for LC3 staining. Over 90% of the starved cells met this criterion. To account for any low levels of autophagy in normal growth media (NGM), cells displaying three or more punctate spots were scored as positive for LC3 staining. Less than 10% of the cells in growth medium had three or more punctate spots. (C) Before plating on LM5, LC3-GFP–infected cells were pretreated with anti-integrin antibodies to α3 (alpha3), α6 (alpha6), or β4 (beta4), or IgG (IgG). After 48 h the number of cells containing 10 or more LC3 punctate spots was counted.

tors, 50 μM butylated hydroxyanisole (BHA) or 1.25 mM *N*-acetylcysteine (NAC). Treatment of PECs with NAC or BHA alone did not significantly increase or decrease the basal level of Annexin V staining. However, pretreatment with either NAC or BHA prevented the induction of Annexin V staining in cells treated with the EGFR inhibitor PD168393 (Figure 7). Thus loss of integrin-mediated signaling through EGFR results in an increase in ROS, which is required for the subsequent induction of caspase-independent death.

DISCUSSION

Using primary cultures of epithelial cells isolated from human PECs, we have identified at least three integrin-mediated signaling pathways whereby adhesion of PECs to their native LM5-rich matrix mediates cell survival (Figure 8). Adhesion of growth factor starved PECs to LM5-rich matrix is required to maintain autophagy. Signaling through $\alpha3\beta1$, and to a lesser extent $\alpha6\beta4$, is required for autophagy. Under starvation conditions cell survival is also dependent on at least two additional independent integrin signaling pathways: 1) integrin-mediated activation of EGFR and subsequent signaling to Erk and 2) integrin-mediated activation of Src, the former being dependent on $\alpha3\beta1$ integrin. Interestingly, there was no activation of the PI-3K/Akt signaling pathway in PECs on LM5; consequently there was no dependence on this pathway for survival in normal PECs. In the presence of an intact autophagy pathway, inhibition of EGFR/Erk or Src is sufficient to induce cell death, but this death is mediated through a caspase-independent mechanism that is dependent on the generation of reactive oxygen species. On the other hand, disruption of autophagy, pharmacologically or by blocking $\alpha3\beta1$, leads to caspase activation and death.

Integrin-mediated transactivation of receptor tyrosine kinases has been widely reported, however, the biological significance of this cross-talk is largely unknown. In this study we demonstrate that EGFR and its ability to activate Erk is a critical pathway for integrin-mediated survival in primary PECs adherent to their native matrix. Previous studies demonstrated that survival of EGFR over expressing

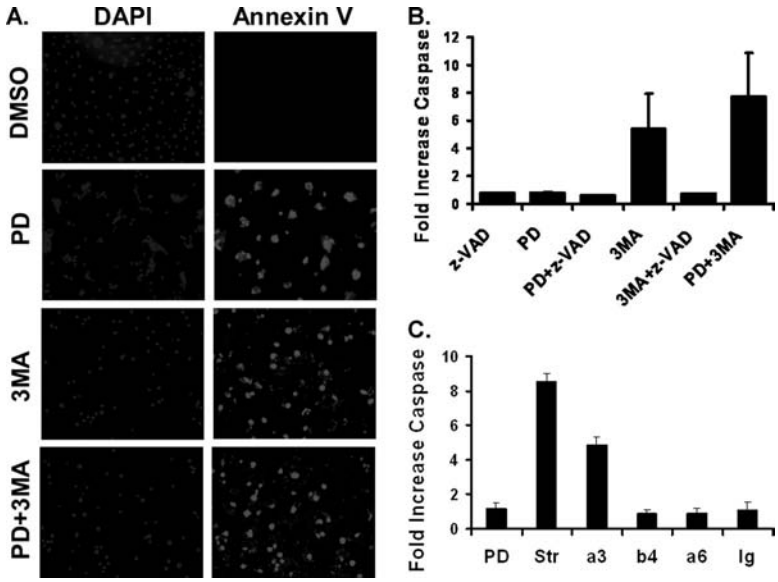

Figure 6. Blocking autophagy induces death and caspases in PECs. PECs were plated on LM5 and treated with DMSO, 0.5 μM PD168393 (PD), 10 mM 3-methyladenine (3MA), or both (PD + 3MA) for 72 h. The caspase inhibitor z-VAD at 20 μM was added at the time of plating as indicated (zVAD). Cells were analyzed for (A) Annexin V staining by fluorescence microscopy and (B) and fold increase in caspase activity relative to DMSO-treated cells. (C) PECs plated on LM5 were treated with DMSO, 0.5 μM PD168393 (PD), 1 μM staurosporine, 10 μg/ml α3 (a3), α6 (a6), β4 (b4) blocking integrin antibodies or IgG (Ig) and the fold increase in caspase activity was measured 48 h later.

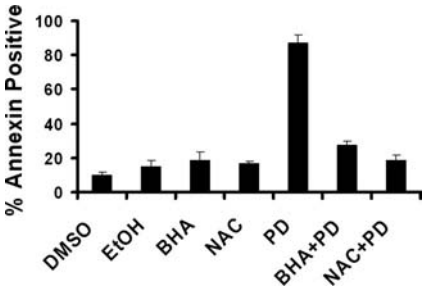

Figure 7. Generation of ROS is required for caspase-independent death induced by EGFR inhibition. PECs were growth factor-starved for 48 h and placed in suspension for 30 min. Cells were allowed to adhere to LM5 in the absence or presence of 50 μM butylated hydroxyanisole (BHA) or 1.25 mM *N*-acetylcysteine (NAC) with or without 0.5 μM PD168393 (PD). Percent Annexin V staining was quantified 72 h later by fluorescence microscopy.

NIH-3T3 cells on fibronectin required integrin-mediated activation of EGFR, but did not involve signaling to Erk, but rather PI-3K (Moro *et al.*, 1998). These differences may reflect the cell type, fibroblasts versus epithelial cells, or the matrix that was used, fibronectin versus laminin. Although LM5 is the predominant matrix secreted by PECs, we cannot rule out the possibility that additional matrix materials may also be present that could be contributing to PEC survival. Nonetheless, we have demonstrated that integrin-mediated signaling through $\alpha3\beta1$ is required for survival mediated by both autophagy and EGFR/Erk signaling on this PEC-generated matrix.

Because PECs rapidly secrete their own LM5-rich matrix, it has not been possible to determine the role of different matrices in regulating long-term survival of PECs. However, in short-term 1-h adhesion assays we have observed that adhesion of PECs to LM1, but not the LM5-rich matrix, is sufficient to activate Akt, suggesting signaling pathways on other matrices may be important in survival. We are currently developing siRNA-based methods for eliminating LM5 from the PEC matrix, which will allow us to investigate the role of EGFR and other signaling pathways in mediating survival on other matrices.

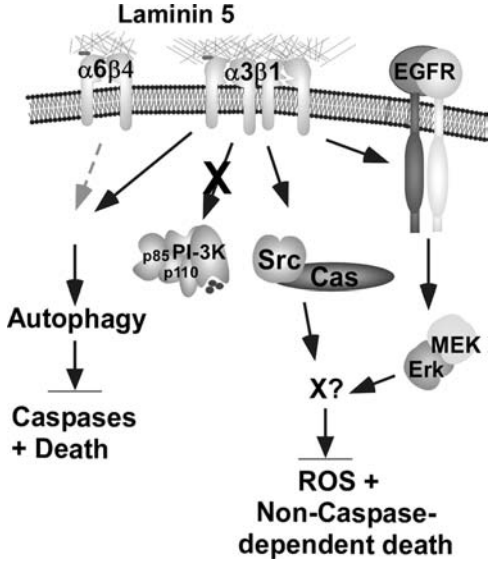

Figure 8. Model for LM5-mediated survival. Adhesion of growth factor-deprived PECs to LM5 via $\alpha3\beta1$ and $\alpha6\beta4$ integrin mediates cell survival by maintaining starvation-induced autophagy. Signaling via $\alpha3\beta1$ to Erk through EGFR or through Src is also required for cell survival. The PI-3K/Akt pathway is not activated on LM5 and not required for survival. Disruption of either the EGFR/Erk or Src pathway leads to caspase-independent cell death, due to the generation of reactive oxygen species (ROS), whereas disruption of autophagy leads to caspase activation and death.

Integrin-mediated survival of primary keratinocytes on LM5 has been shown to involve signaling to Erk (Manohar *et al.*, 2004). As in our studies, keratinocyte survival on LM5 was dependent on $\alpha3\beta1$ integrin. Whether Erk activation in keratinocytes is dependent on integrin signaling through EGFR has not been reported. However, autocrine ligand-mediated signaling through EGFR to Erk was shown to contribute to cell survival of keratinocytes in suspension (Jost *et al.*, 2001). We have ruled out a role for autocrine ligand involvement in PECs, because ligand binding blocking antibodies do not block integrin-mediated EGFR activation or downstream signaling to Erk. Given the similar findings in primary prostate epithelial cells and keratinocytes, we predict that keratinocyte survival on LM5 should also involve integrin-mediated activation of EGFR and subsequent downstream signaling to Erk.

Whether integrin-mediated activation of other receptor tyrosine kinases is involved in regulating survival on matrix is not known. However, it was recently demonstrated that ligand-independent activation of c-Met in PC3 cells was required for cell survival (Shinomiya *et al.*, 2004). The specific integrins, matrix, and signaling pathways involved in c-Met–mediated survival are currently unknown. Furthermore, whether c-Met regulates integrin-mediated survival in normal primary PECs is also unknown. Our data indicate that PC3 cells, which express low levels of EGFR relative to PECs, do not activate EGFR or Erk upon integrin engagement and do not depend on this pathway for integrin-mediated survival. Instead survival of PC3 cells requires PI-3K and Src. c-Met is known to activate these signaling pathways in response to HGF, but whether c-Met participates in integrin-mediated signaling to PI-3K or Src in PC3 cells has not been determined.

Surprisingly, interference with integrin signaling through EGFR/Erk or Src leads to caspase-independent death. This was unexpected, because cell death due to anoikis has been reported to be caspase-dependent. Our results suggest that the mechanism of cell death induced during complete loss of cell adhesion through integrins is different from the mechanism of cell death induced by interfering with specific signaling downstream of integrin engagement. One possible explanation is that loss of attachment is likely to interfere with several different signaling pathways simultaneously, whereas our studies individually dissected distinct pathways. In fact, blocking $\alpha3\beta1$ alone was sufficient to induce caspase activity in PECs, similar to what has previously been observed in keratinocytes derived from integrin $\alpha3$ null mice (Manohar *et al.*, 2004). We have demonstrated that at least one of the signaling pathways activated by $\alpha3\beta1$ is EGFR/Erk. Src activation is also important for integrin-mediated survival, but inhibition of both EGFR and Src was also not sufficient to induce caspases, indicating additional survival pathways are involved.

Previous studies in mammary epithelial cells demonstrated that cells in the center of acinar structures undergo autophagy and die during morphogenesis (Melino *et al.*, 2005). However, the current prevailing theory on the primary role of autophagy is to promote temporary survival under growth factor and nutrient deprivation conditions,

rather than to be a direct mechanism for programmed cell death (Lum *et al.*, 2005). Interestingly, inhibiting autophagy in LM5-adherent PECs induced caspase activation and cell death and did not rescue cells induced to die by inhibition of EGFR/Erk, suggesting that cell death was not dependent on autophagy. Thus adhesion of starved PECs to a LM5-rich matrix induces an autophagic state that permits survival, but further assault by inhibiting EGFR/Erk activation leads to caspase-independent death.

Blocking $\alpha3\beta1$ integrin significantly reduced the extent of autophagy induced under starvation conditions. Thus, in addition to regulating EGFR/Erk, $\alpha3\beta1$ integrin is also required to maintain autophagy. Although blocking $\alpha6\beta4$ integrin also reduced autophagy, the effect was not as dramatic as blocking $\alpha3\beta1$. Furthermore, $\alpha6\beta4$ was not required for EGFR/Erk activation and disruption of this integrin alone failed to induce caspase activation or cell death. Therefore, the small reduction in autophagy seen by blocking $\alpha6\beta4$ may not be sufficient enough to overcome other survival pathways that are active in the cell, such as EGFR/Erk and/or Src. Interestingly, inhibition of both autophagy and EGFR signaling lead to a small (although not statistically significant in the four assays examined), but consistent increase in caspase activation, suggesting that in the absence of autophagy signaling through EGFR/Erk may still contribute to cell survival. One possibility is that signaling through EGFR/Erk helps to maintain autophagy. However, if this is true, then there must be other pathways involved, because inhibition of EGFR/Erk alone is not sufficient to induce caspase-mediated death.

It is interesting to note that signaling through PI-3K is actually inhibitory to the development of autophagy (Rusten *et al.*, 2004). Adhesion of PECs to LM5 does not activate the PI-3K pathway. This suggests that the absence of strong PI-3K signaling permits the survival of PECs on LM5 through autophagy. It is striking that cell death induced by loss of EGFR/Erk or Src signaling is not sufficient to activate caspases. This suggests the existence of a strong anticaspase mechanism present in PECs. Recent studies have suggested that the presence of an autophagic state can be inhibitory to the activation of caspases (Degenhardt *et al.*, 2006; Abedin *et al.*, 2007). One model proposes that the autophagy pathway selectively targets damaged mitochondria for destruction by walling them off from the cytoplasm and thus preventing the release of enzymes required for the induction of caspases. Therefore, it is possible that the absence of a PI-3K pathway may allow this shift to a caspase inhibitory state. Thus in the EGFR/Erk inhibited cells, caspase-mediated death is dominantly inhibited, forcing other death mechanisms to be activated when this level of stress is induced.

Many human prostate cancers have reduced levels of the negative PI-3K regulator, Pten, and the PI-3K/Akt pathway is constitutively activated in those tumors (McMenamin *et al.*, 1999). Furthermore, the development of prostate cancer is accompanied by the loss of LM5 in the basement membrane (Davis *et al.*, 2001). Therefore, given that autophagy is driven by LM5-mediated adhesion and suppression of PI-3K signaling, we would predict that loss of LM5 and increased PI-3K signaling would prevent the induction of an autophagy survival pathway in tumor cells and make them more sensitive to caspase-mediated death.

Several caspase-independent mechanisms of cell death have been described, including activation of cathepsins, calpeptins, ROS, and release of numerous destructive enzymes from the mitochondria (Kroemer and Martin, 2005). To date we have been unable to detect release of cytochrome C from mitochondria in PECs treated with EGFR inhibitors, and inhibition of calpeptin did not rescue the death induced by inhibiting EGFR/Erk signaling (not shown). However, by blocking the generation of ROS with two different inhibitors, we were able to prevent the cell death induced by inhibition of EGFR/Erk. Thus adhesion to matrix and signaling through EGFR/Erk may act to limit ROS production. Integrin $\alpha1$ null mesangial cells have been reported to have enhanced ligand-independent EGFR activation and excessive ROS production, suggesting that integrin signaling can help to modulate EGFR activation and limit ROS production (Chen *et al.*, 2007). Another report suggests that $\alpha1$ negatively regulates EGFR activation by stimulating the activity of TC-PTP (Mattila *et al.*, 2005). However, if this same mechanism is acting in PECs, then loss of EGFR/Erk signaling would lead to reduced ROS production rather than its increase. Thus the mechanism by which loss of EGFR/Erk signaling leads to enhanced ROS production is not clear. Interestingly, a recent report indicates that high levels of ROS are required for starvation-induced autophagy (Scherz-Shouval *et al.*, 2007). Therefore it is possible that one side effect of EGFR/Erk inhibition may be to further enhance autophagy through increased generation of ROS.

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